2018

Dually Modified Transmembrane Proteoglycan TβRIII/Betaglycan In Cell Signaling And Cancer

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DUALLY MODIFIED TRANSMEMBRANE PROTEOGLYCAN TβRIII/BETAGLYCAN IN CELL SIGNALING AND CANCER

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Submitted in Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy in

Chemistry

College of Arts and Sciences

University of South Carolina

2018

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DEDICATION

This dissertation is dedicated to my parents, sister and fiancé. Thank you for your endless love, support and encouragement.

ACKNOWLEDGEMENTS

I am grateful to my advisor Dr. Mythreye Karthikeyan for providing the support, guidance, patience and enthusiasm needed to guide my scientific journey. I thank her for providing me with the freedom to explore and learn from my successes and failures while continuously helping me understand my research and myself more and more each day. The training and strong scientific background that I have developed while in her lab will serve me well as I pursue my scientific passions in the future. I am also grateful to Anne Serrao, Ben Horst, Pratik Patel and my undergraduate team (Haley F., Shreya S., Carly L., Mariah H. and Juliet J.) for their continued support of my research and passions. I would also like to thank my extraordinary thesis committee as they have guided me along this process by giving me constructive criticism and guidance.

This work would not have been possible without the financial support of the following organizations and I would like to personally thank them for their support over the years: National Institutes of Health (NIH) (Grants P20-GM109091 and GM122379-01), Marsha Rivkin Foundation, USC SPARC Graduate Research Program, USC Center for Colon Cancer Research and the American Association of University Women.

To my family, friends and fiancé: Thank you. Thank you for your dedication to my career in science and support of my dreams. I could not have done any of this without you all. You all have been continuous sources of love, motivation and encouragement for me. Thank you parents for being such excellent role models for Brittany and I and for being our biggest cheerleaders. Thank you Brittany for keeping me motivated and “on my

toes." It's an honor to be your older sister. Finally, thank you Jeremy for being my rock. You know how to be a friend, critic, problem-solver and listener and you have truly helped me see my goals to the end. Thank you everyone, once again.

ABSTRACT

During tumor progression, cancer cells undergo a number of alterations to evade tumor inhibitory mechanisms, proliferate, invade surrounding tissues and metastasize to distant sites. These properties reflect changes in their cell signaling pathways that, in normal cells, control aberrant cell proliferation, motility, and survival. Over the past few decades, hyperactive Wnt/β-catenin signaling has been linked to the formation of multiple cancers, including malignant ovarian cancer, making identification of molecules regulating Wnt/β-catenin signaling crucial to the development of early detection methods and cancer treatment strategies. Proteoglycans, which regulate transmission of cellular signals, are implicated in the pathophysiology of diseases, including cancer, where signals and tissue interactions malfunction. These cell surface molecules consist of a core protein decorated extracellularly with covalently linked heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate and/or keratan sulfate glycosaminoglycan (GAG) chain modifications. A small group of proteoglycans that contain the two most common GAG chain attachments, HS and CS chains, span the cell membrane to regulate signal transduction and are hereby referred to as dually modified transmembrane proteoglycans (DMTPs). Two examples of DMTPs are betaglycan (TβRIII) and syndecan-1 (SDC1). These DMTPs play important roles in modulating key cell signaling pathways to affect epithelial cell biology and cancer progression. The overall goal of my dissertation research was to determine the impact of betaglycan, a well-established TGFβ co-receptor, on Wnt/β-catenin signaling. To this end, my work has revealed new roles for betaglycan

in Wnt signaling. Specifically, depending on its GAG chain modification, betaglycan can either enhance or suppress canonical Wnt/β-catenin signaling. My findings implicate interactions between Wnt and betaglycan's HS chains in inhibiting Wnt signaling, likely via Wnt sequestration, while the CS GAG chains on betaglycan promote Wnt signaling. My studies reveal a novel, dual role for TβRIII/betaglycan and define a key requirement for the balance between CS and HS chains in dictating ligand responses. Ultimately, my findings will help unravel the complex roles that DMTPs and their GAG chains play in regulating ligand availability and cell signal transduction during disease progression.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACTR ..... Activin Type Receptor

ALK ..... Activin Receptor-like Kinase

BMP ..... Bone Morphogenetic Protein

BMPR ..... Bone Morphogenetic Protein Receptor

CS ..... Chondroitin Sulfate

DMTP ..... Dually Modified Transmembrane Proteoglycan

ECD ..... Extracellular Domain

ECM ..... Extracellular Matrix

EMT ..... Epithelial-Mesenchymal Transformation

FGF ..... Fibroblast Growth Factor

FGFR ..... Fibroblast Growth Factor Receptor

FZD ..... Frizzled

GAG ..... Glycosaminoglycan

HSPG ..... Heparan Sulfate Proteoglycan

HS ..... Heparan Sulfate

LRP ..... Low-density Lipoprotein Receptor (LDLR)-related Proteins

MAPK/ERK ... Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase

MMP ..... Matrix Metalloproteinases

PDZ ..... Postsynaptic Density-95/Disc Large Protein/Zonula occludens-1

R-SMAD ..... Receptor-regulated Smad

sBetaglycan ..... Soluble/Shed Betaglycan

SDC1 ..... Syndecan-1

sDMTP ..... Soluble Dually Modified Transmembrane Proteoglycan

sSDC1 ..... Soluble/Shed SDC1

TβR ..... TGF-β Receptor

TGF-β ..... Transforming Growth Factor β

TP ..... Transmembrane Proteoglycans

WNT ..... Wingless/Integrated

ZP ..... Zona Pellucida

CHAPTER 1

INTRODUCTION TO DUALLY MODIFIED TRANSMEMBRANE PROTEOGLYCANS (DMTPS)[1]

[1] Jenkins, L. M., Horst, B., Lancaster, C. L., and Mythreye, K. (2017). Dually modified transmembrane proteoglycans in development and disease. *Cytokine & growth factor reviews.* In press.

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Transmembrane proteoglycans (TP) are members of the heparan sulfate proteoglycan (HSPG) family whose core domains span the cell membrane to modulate a multitude of intracellular and extracellular activities(1-10). TP extracellular domains (ECD) contain covalently attached glycosaminoglycan (GAG) chain modifications that include heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate and/or keratan sulfate, with HS and CS chains being the most common(2). As members of the GAG family, HS and CS chains are characterized by repeating disaccharide units containing glucuronic acid and either N-acetylgalactosamine for HS or N-acetylglucosamine for CS(1). Of the known human transmembrane proteoglycans, only a few can present both HS and CS chains and are referred to here as dually modified transmembrane proteoglycans (DMTP). Human DMTP members include the Type III Transforming Growth Factor β Receptor/Betaglycan, Syndecans-1 and -3, CD44 and Neuropilin-1. Syndecan-3 (SDC3), similar to betaglycan and syndecan-1 (SDC1), contains both CS and HS chains but is found almost exclusively in neuronal and musculoskeletal tissue(11) with defined roles in skeletogenesis(12) and neurite growth(13). Unlike SDC3, CD44 is expressed in many different cell types(14) and in various isoforms, which can exhibit HS and/or CS chains depending on the splice variant(2,15-18). In epithelial cells, the primary form of CD44, known as CD44E or CD44v8-10, lacks the variable region (exon v3) required for HS chain attachment and therefore can only express CS chains(14,18-21). Neuropilin-1 (NRP1), which is prominent in neuronal and endothelial cells, has clearly outlined roles in axonal growth/guidance as well as physiological and pathological angiogenesis(22-24). Since NRP1 only contains one serine residue (Ser^{612}) for GAG chain attachment, it can only exist as a HS proteoglycan or a CS proteoglycan but not as a

hybrid of the two(24-27). DMTPs are present in both membrane-bound and/or shed/soluble forms with these forms able to impact cell behavior by either mimicking or opposing each other's cellular functions. Contributions from the core domain's scaffolding properties can also influence GAG chain-ligand binding(7), highlighting the importance of the HSPGs' core domain in delineating roles for GAG chains. Consequently, shed/soluble DMTPs and their attached GAG chains can affect ligand availability, downstream signaling and promote interactions between GAG chains, ligands and the extracellular environment(28).

The background summarizes regulatory roles for both betaglycan and SDC1 in growth factor signaling with the dissertation work focused primarily on betaglycan in Wnt signaling. I emphasize the underappreciated mutual dependence of HSPGs' GAG chains and core protein and their impact in disease, with a focus on cancer progression. Finally, I provide comprehensive information on these DMTPs for the future development of therapeutic techniques to modulate growth factor/ligand availability and thus control downstream cell signaling pathways involved in human disease.

1.1 DMTP Structure and Features

1.1.i. Core Structure

Both SDC1 and betaglycan are proteoglycans containing a large ECD, a transmembrane domain and a short cytoplasmic domain (~200 amino acids or less) that lacks intrinsic kinase activity. Betaglycan's ECD contains a zona pellucida domain (ZP1 and ZP2, Figure 1.1), which is important for protein polymerization and protein-protein interactions(29). Specifically, betaglycan's ZP domain contains two distinct Transforming Growth Factor β (TGF-β) binding sites: one in the membrane-distal region

(ZP1)(Figure 1.1 and (30)) and one in the membrane-proximal ZP region (ZP2)(Figure 1.1 and (31)). TGF-β ligands TGF-β1/2/3, Bone Morphogenetic Proteins (BMPs) 2/4/7 and Growth Differentiation Factor 5 (GDF-5) can bind both regions while the TGF-β member Inhibin only binds the ZP region (ZP2)(32,33). Unlike betaglycan, little is known about SDC1's ectodomain core independent of its GAG chain functions. SDC1's 235 amino acid(34) extracellular core, however, does support fibroblast cell adhesion(35), interacts with integrins αvβ3 and αvβ5 during angiogenesis and αvβ1 integrin during re-epithelialization of lung tissue(36-40).

The short cytoplasmic domains of DMTPs are serine/threonine rich, frequently phosphorylated and contain a PDZ (Postsynaptic Density-95/Disc large protein/Zonula occludens-1) binding motif at the extreme carboxy terminus. These cytoplasmic regions commonly interact with scaffolding proteins to control DMTP internalization and cell signaling (Figure 1.1 and (2,34,41,42)). Betaglycan's short cytoplasmic domain, through its Class I PDZ binding motif, binds the scaffolding protein GAIP-interacting protein C terminus (GIPC) to modulate TGF-β signaling (43).

Additionally, the Type II TGF-β Receptor (TβRII) can phosphorylate betaglycan's cytoplasmic domain at Thr^{841} to promote β-arrestin2-betaglycan interactions and receptor internalization (44). SDC1's cytoplasmic domain is divided into two conserved regions, C1 and C2 (Figure 1.1), which are common regions between all syndecans, and a central variable region (V, Figure 1.1), which confers specific properties on each syndecan (Figure 1.1 and (45)). SDC1's V domain regulates cell spreading and actin cytoskeleton assembly(46) while its C1 domain, adjacent to the membrane, participates in SDC1 dimerization and intracellular protein binding(47). The conserved

C2 carboxyl-terminal tetrapeptide sequence can bind PDZ-domain-containing proteins(41,42), which may function as membrane scaffold proteins that recruit signaling and cytoskeletal proteins to the plasma membrane. Contributions from the core domain's scaffolding properties are likely to influence GAG chain-ligand binding by either immobilizing the chains to specific sites within the plasma membrane(7) or by altering the internalization of ligands bound to GAG chains (48). Additionally, the position of the GAG chains on the core protein (plasma membrane distal or proximal) could impact GAG chain association with receptors and subsequent DMTP function.

1.1.ii. GAG Chain Structure

On the extracellular core of DMTPs are sites for linear polysaccharide GAG chain attachments (Figure 1.1 and (1,2,30,34,49)). Synthesis of DMTP GAG chains begins with covalent attachment of a linkage tetrasaccharide, glucuronic acid-galactose-galactose-xylose (GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-O-), to a serine hydroxyl group on the core protein followed by addition of repeating disaccharide regions composed of hexosamine and hexuronic acids (Figure 1.1 and (49)). The repeating disaccharide units distinguish HS chains from CS chains. During initial synthesis of HS chains, a N-acetylglucosamine (GlcNac) is attached to the linkage tetrasaccharide by α1,4-N-acetylglucosaminyltransferase-I (α4GlcNAcT-I). On the other hand, synthesis of the CS chains occurs when β1,4-N-acetylgalactosaminyltransferase-I (β4GalNAcT-I) attaches a N-acetylgalactosamine disaccharide to the linkage tetrasaccharide(49). The first hexosamine residue thus determines which chain is made (HS or CS). After attachment of this fifth saccharide, the chondroitin synthase family (for CS chains) or exostosins (for HS chains) attach the remaining disaccharide units(49). HS chains can then be selectively

sulfated at the *N, 3-O* and *6-O* positions of glucosamine and the *2-O* position of uronic acid residues by sulfotransferases(50) while sulfate groups on CS chains are added at *C-4* and/or *C-6* of *N*-acetyl-galactosamine and/or *C-2* of glucuronic acid(51). In vertebrates, SDC1 is one of four syndecan family members and is the primary syndecan expressed in epithelial cells(52). As a "full-time" proteoglycan (no GAG-less form *in vivo*), SDC1's ECD contains three attachments sites for HS chains (Ser^{37}, Ser^{45} and Ser^{47}) and two sites for CS chain attachment (Ser^{210} and Ser^{220})(Figure 1.1 and (34,53,54)). While SDC1 is an obligate or "full-time" DMTP, betaglycan is commonly referred to as a "part-time" proteoglycan since it can be expressed with or without GAG chains(1,2,49,55) *in vivo*. CS chains attach to betaglycan's 100 kDa protein core primarily at Ser^{545}. CS chains can also attach at Ser^{534}, but HS chains usually predominate at this site (Figure 1.1 and (30,49,56)). Both alterations in core protein expression and GAG chain density and/or composition (HS vs. CS) can impact DMTP functions.

1.1.iii. Shedding and Intracellular Domain (ICD) Cleavage

Several DMTP functions can be attributed to ectodomain shedding which leads to DMTP expression in both membrane-bound and shed/soluble forms. Both forms can impact whether GAG chains are anchored to the membrane or present as soluble GAGs in the extracellular matrix (ECM). Cleavage occurs near the transmembrane domain to release a soluble ectodomain containing GAG chains (Figure 1.1). This ectodomain shedding is highly regulated by the enzymatic activity of several proteases(2,39,57-61) including matrix metalloproteinases (MMP) and metalloprotease disintegrins. Enzymatic activity by pervanadate, a general tyrosine phosphatase inhibitor, stimulates cleavage of both SDC1(62) and betaglycan(59). Sheddases Membrane type MMP-7(63,64),

matrilysin(65), MMP-9(66) and MMP-1(60) cleave SDC1's ECD. Intriguingly, shedding for SDC1 has also been shown to be regulated by intracellular interaction of the small GTPase Rab5 with SDC1's intracellular domain as Rab5's dissociation from SDC1's cytoplasmic domain triggers SDC1 ectodomain shedding at the cell surface(63). It is postulated that activated GTP-Rab5 dissociates from SDC1's cytoplasmic domain, associates with β1 integrin and stimulates the internalization of β1 integrin and dissociation of β1 integrin from SDC1. This cascade of events exposes SDC1's ECD cleavage site to sheddases(63).Whether such intracellular control of betaglycan shedding exists has not been tested. However, the ECD of betaglycan is cleaved by MMP-1 and -3(59). Both SDC1 and betaglycan's transmembrane/cytoplasmic fragments can be cleaved by γ-secretase to release both DMTPs' cytoplasmic domains intracellularly (Figure 1.1 and (67,68)). Functions for these cytoplasmic domains are reviewed in (67,68).

Expression of different GAG chains on membrane bound DMTPs can impact soluble DMTP (sDMTP) pools, as observed with membrane bound SDC1's HS chains, where higher membrane bound HS modified SDC1 leads to lower SDC1 shedding(69). Altering the balance of membrane bound versus soluble DMTPs could, in effect, impact cell signaling by altering ligand interactions with sDMTPs' ECD/GAG chains (See Chapters 2-4). In the cell media, for example, increased levels of sDMTPs containing HS chains exclusively could suppress signaling as they may act as ligand traps capable of reducing ligand availability to the membrane-bound cognate receptors. Cleaved DMTP ectodomains containing CS chains could prevent ligand aggregation in the cell media, thereby facilitating ligand-receptor interaction and increasing cancer cell signaling. Given

the current impact of DMTPs in regulating cell signaling, as outlined in Chapters 2-4, an in-depth analysis of the GAG chain composition on membrane-bound and sDMTPs may clarify roles for DMTPs and their GAG chains in modulating growth factor signaling and cell biology.

1.2. DMTPs in Development

SDC1 is expressed during development to modulate tissue morphogenesis as well as during wound healing, with several of its developmental functions mediated by its GAG chains' interactions with a number of extracellular adhesion proteins and growth factors(46,52,70). SDC1 interaction with laminin, for example, is completely dependent on both its HS and CS chains and leads to increased cell adhesion to the basement membrane(71). However, SDC1's CS chains promote Slit signaling to drive axon and myotube guidance when bound to the core protein(28,72), suggesting differential roles for the independent GAG chains. Betaglycan's significance in development is underscored by the observed embryo lethality in betaglycan knockout mice after 13.5 days(73,74). This lethality is partly due to significant impairment of coronary vasculogenesis in betaglycan-null mice, demonstrating a requirement for betaglycan in coronary vessel development and embryonic viability(74). Additionally, betaglycan-specific antisera inhibits mesenchyme formation and endothelial cell migration in the atrioventricular (AV) cushion, where cardiac endothelial cells lining the lumen of the AV cushion undergo epithelial-mesenchymal transformation (EMT) to participate in valve and membranous septa formation(75-77). Moreover, betaglycan overexpression in non-transforming ventricular endothelial cells confers transformation in response to TGF-β2, suggesting betaglycan promotes heart cell transformation via TGF-β signaling(76). As

part of the adaptive immune response, T-cell receptors (TCRs), which are expressed on T lymphocytes, recognize antigens associated with molecules of the major histocompatibility complex(78). In both lymphoid and stromal cells of the thymus, betaglycan has been shown to promote T-cell development by protecting thymocytes from apoptosis(79). Individual functions for betaglycan's HS and CS chains during development are still under investigation.

1.3. DMTPs in Disease

Over the past few decades, an increasing body of evidence suggests that HS and CS chains heavily influence cell biology and cancer progression(80). GAG chains, which are present at the cell surface of almost all tumor cells(80), can regulate growth factor signaling, tumor cell survival, angiogenesis, growth, invasion and metastasis(80). HSPGs can exhibit both pro-tumorigenic(3-6) and anti-tumorigenic effects depending on their GAG modifications(7-10). In addition to cancer, changes in HSPG expression/function are also observed in other human diseases, including arthritis, inflammation(3), Alzheimer's/dementia(81), osteoporosis(82), fibrosis(83) and ischemic heart disease(84). Furthermore, HSPGs have been shown to alter wound healing and matrix remodeling, outcomes that can impact a broad range of pathologies(3,73,84,85), making our understanding of HSPG/GAG chain roles in the context of the cellular environment critical. Since much of the research emphasis has been placed on HSPG/GAG chain roles in cancer (>8% of all PubMed articles), ***my dissertation work will focus on summarizing and analyzing betaglycan's GAG chain roles in Wnt signaling as it relates to cancer.***

1.3.i. DMTPs in Cancer

Both SDC1 and betaglycan have established roles in regulating tumor progression with specific roles in apoptosis, adhesion, angiogenesis, proliferation, migration, invasion and metastasis *in vitro* and *in vivo*(2,9,39,52,86-96). The membrane-bound form of SDC1 is capable of suppressing apoptosis as SDC1 knockdown in myeloma cells induce growth arrest and apoptosis(4). Other reports, however, indicate that recombinant human SDC1 ectodomains induce apoptosis in breast cancer cells(8). Whether differential GAG modifications in the recombinant SDC1 ectodomains versus SDC1 in myeloma account for the differences observed is unclear. Moreover, while increased SDC1 levels correlate with a more favorable prognosis in mesothelioma, head and neck, pancreatic, lung and invasive breast cancers(2,3,39,97,98), both tumor promoting and tumor suppressing effects have been noted in colorectal and prostate cancers(39). These seemingly contradictory functions for SDC1 emphasize the variability of DMTP roles in cancer. Similarly, in the case of betaglycan, loss or reduced expression has been demonstrated in tumors(86-88,91,99). In particular, betaglycan expression has been shown to be a strong predictor of overall and recurrence-free patient-survival in certain cancers, such as breast cancer and neuroblastoma(86,90,100). Although betaglycan expression is commonly lost in most tumor types, acting primarily as a tumor suppressor(2,86-88,90,100), betaglycan expression has been shown to be elevated in colon cancer, a subset of triple negative breast cancers and high-grade lymphomas, indicating context dependent functions for betaglycan(5,6,101). Whether or not betaglycan's differential role in cancer can be attributed to its HS and CS chain functions remains to be determined.

In addition to controlling cell signaling in an autocrine fashion, sSDC1 can also

deliver growth factors in a paracrine manner to neighboring cells in the environment. sSDC1 from multiple myeloma cells, for example, transports hepatocyte growth factor (HGF) to bone marrow stromal cells to regulate gene expression(39,102). These HGF/sSDC1 interactions in the ECM likely cooperate with already established roles for membrane SDC1 in promoting myeloma cell growth, survival and spread through HS chains interactions with HGF(3). Betaglycan levels in the tumor stroma may vary depending on the tumor type. For instance, betaglycan expression is significantly higher in the stroma of early-stage, stroma-rich neuroblastomas(100), yet is down regulated in the tumor stroma of breast tumors(103). In neuroblastoma, increased stromal expression confers a significant survival advantage as patients with increased stromal betaglycan exhibited significantly higher event-free survival due, in part, to increased Fibroblast Growth Factor (FGF) signaling and differentiation(100). Contrastingly, in breast tumor stroma, decreased betaglycan mRNA and protein expression is linked to poorer patient outcome(103), supporting betaglycan's contextual roles in cancer. Whether stromal cells can take up sBetaglycan from tumor cells to impact gene expression, like in the case of sSDC1, has not been determined. Taken together, these data suggest that DMTPs can have important effects on cancer progression as potent regulators of tumor stromal interactions mediated partly through their GAG chains.

In the tumor environment, cancer cells can subvert the host immune system with mechanisms similar to those they use to evade immunotherapeutic strategies(104-106). Additionally, effector cell dysfunction and an increased number of regulatory/suppressor T-cells in cancer patients may further limit the efficacy of immunotherapeutic approaches(107). In murine and human lymphocyte subpopulations, betaglycan is

significantly and preferentially expressed on naïve and central memory T-cells and is upregulated upon TCR stimulation. Treating cells with sBetaglycan decreased regulatory/suppressor FoxP3^{+} T-cell generation, in a TGF-β-dependent manner, suggesting a promoter role for betaglycan in T-cell tolerance and immune responses(78). In both murine breast cancer and melanoma, progressive loss of betaglycan expression generates an immunotolerant tumor microenvironment whereby TGF-β signaling is enhanced and upregulates both immunoregulatory enzyme indoleamine 2,3-dioxygenase in plasmacytoid dendritic cells (DCs) and the CCL22 chemokine in myeloid DCs(104). This mechanism, coupled with the elevated TGF-β levels, creates an immunotolerant environment and may facilitate tumor progression and metastasis(104). Contrary to betaglycan's ability to increase tumor sensitivity to immune defenses, SDC1 appears to aid in cancer's subversion of the host immune system. For example, myeloma cells coated with anti-SDC1 antibodies displayed enhanced cross-presentation of tumor-derived cellular antigens and generated tumor specific–killer T cells by DCs(108), suggesting an immunosuppressive role for SDC1. Additionally, anti-SDC1 immunotoxin (IT, B-B4-SO6), in combination with the chemotherapeutic drug doxorubicin, led to increased cell death in doxorubicin-sensitive multiple myeloma cells(109), further supporting SDC1's tumor promoter role. An in-depth analysis of DMTPs and their GAG chains in modulating interactions between the immune system and malignant cells is warranted as it could strongly augment the design of clinically more efficient immunotherapy strategies.

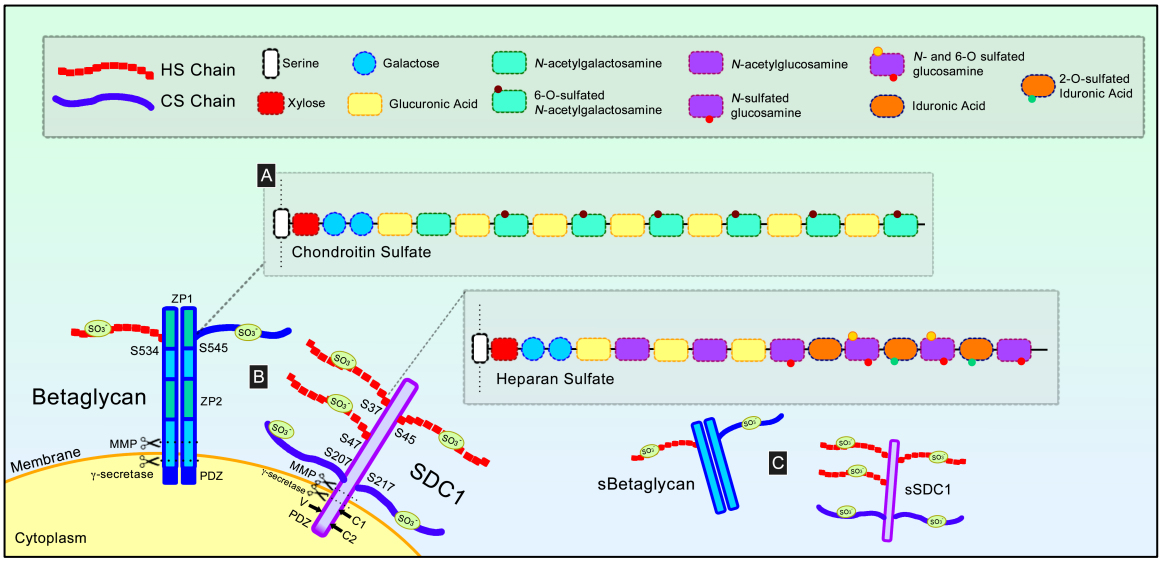

Figure 1.1. Structure and features of DMTPs betaglycan and SDC1. (A) Structures of DMTP CS (top) and HS (lower) glycosaminoglycan (GAG) chains. Both GAG chains share a common linkage tetrasaccharide covalently attached to a serine hydroxyl group on the DMTP core protein. These serine residues have an adjacent glycine residue on the carboxy-terminal side and usually lie within an area rich in acidic residues. (B) Betaglycan's core protein contains two ZP domains in its ECD for ligand binding and both DMTPs contain a small cytoplasmic domain, which can be cleaved by γ-secretase to release the cytoplasmic fragment. A PDZ motif in betaglycan's cytoplasmic domain is frequently phosphorylated and regulates betaglycan cell surface expression and intracellular protein interactions. SDC1's cytoplasmic domain is divided into two conserved regions and a variable region, which are responsible for intracellular protein binding. SDC1 can also interact with proteins containing a PDZ motif. Betaglycan and SDC1 both undergo ectodomain shedding by MMPs to release (C) soluble forms of each receptor, which can both bind growth factors and modulate their activity. Abbreviations: C1: Conserved Domain 1, C2: Conserved Domain 2, MMP: Matrix Metalloproteinases, PDZ: Postsynaptic Density-95/Disc large protein/Zonula occludens-1, sBetaglycan: Soluble/shed betaglycan, sSDC1: Soluble/shed SDC1, V: Variable Domain, ZP: Zona Pellucida, HS: Heparan Sulfate, CS: Chondroitin Sulfate.

CHAPTER 2

WNT SIGNALING REGULATION BY BETAGLYCAN[2]

[2] Jenkins, L. M., Singh, P., Varadaraj, A., Lee, N. Y., Shah, S., Flores, H. V., O'Connell, K., and Mythreye, K. (2016) Altering the Proteoglycan State of Transforming Growth Factor beta Type III Receptor (TbetaRIII)/Betaglycan Modulates Canonical Wnt/beta-Catenin Signaling. *The Journal of biological chemistry* **291**, 25716-25728.

2.1. Introduction

Wnt glycoproteins regulate three distinct Wnt signaling pathways to mediate cell fate, proliferation and apoptosis as well as cancer initiation and progression in multiple cancers, including ovarian (110-118). Activation of the canonical Wnt/β-catenin pathway begins with binding of Wnt to its cell surface receptors, Frizzled and low-density lipoprotein receptor (LDLR)-related proteins LRP5/6, followed by phosphorylation of LRP5/6, recruitment of Dishevelled (Dsh) to the plasma membrane to interact with Frizzled and stabilization of cytosolic β-catenin (119). Axin interaction with phosphorylated LRP5/6 and Dsh leads to inactivation of the β-catenin destruction complex, accumulation of β-catenin and translocation to the nucleus to regulate Wnt target genes by binding to TCF/LEF transcription factors (119,120). The Wnt signaling cascade is controlled, in part, by transmembrane proteoglycans, which interact with Wnt signaling components and can either stimulate or inhibit signaling activity. For instance, the HSPGs Glypican-3 and Syndecan-1 stimulate canonical Wnt signaling (121,122) while others, including Glypican-1 and Glypican-6 suppress Wnt signaling (122,123).

The Type III TGF-β receptor (TβRIII)/Betaglycan is a transmembrane proteoglycan with loss resulting in embryonic lethality in mice (73). Beyond its roles in regulating TGF-β signaling, betaglycan also controls several other pathways to inhibit cell migration, invasion, cell growth and angiogenesis in both *in vitro* and *in vivo* cancer models (2,86-91) and regulating differentiation through FGF2 signaling (9). Mechanistically, betaglycan regulates these pathways by either altering the actin cytoskeleton, via betaglycan/β-arrestin2 cytoplasmic interactions (124), or through GAG chain interactions with FGF2 (9). Overall, betaglycan also acts as a tumor suppressor in prostate (89), lung (125), pancreatic (88) and breast cancer (86,90,93,95) but has been shown to promote metastasis in specific mesenchymal-stem like breast cancers (6) indicating complex roles for betaglycan in cancer.

Although betaglycan's core can bind TGF-β superfamily members with high affinity (30,44,91) the extracellular domain also contains two sites of heparan and chondroitin sulfate GAG chain modifications resulting in betaglycan existing in multiple forms *in vivo* (30,126,127). Given that Wnt glycoproteins have a high affinity for both heparan and chondroitin GAG chains on proteoglycans (50,122), we initiated studies to determine the possible role of betaglycan on canonical Wnt3a signaling.

We find, using both cancer and normal epithelial cells and a combination of loss and gain-of-function approaches, that betaglycan suppresses Wnt3a signaling both at the signal reception level and through inhibition of β-catenin transcriptional activity by binding Wnt3a via its sulfated GAG chains. In contrast, betaglycan's chondroitin sulfate chains can promote Wnt3a signaling, suggesting that the composition of GAG chains may significantly alter cellular response to betaglycan and thereby Wnt signaling. Consistent with a lack of a role for betaglycan's GAG chains in betaglycan's functions as a TGF-β co-receptor (30), betaglycan's suppression of canonical Wnt3a signaling is independent of TGF-β signaling and independent of betaglycan's cytoplasmic domain interactions described previously (43,124,128). These results demonstrate an intricate mode of Wnt3a signaling regulation by betaglycan, mediated largely by its heparan and chondroitin chains, and lay the foundation to advance current understanding of the various roles proteoglycans, with different GAG chains, can have in maintaining cellular homeostasis, specifically through control of Wnt availability and signaling.

2.2. Experimental Procedures

Cell Lines and Reagents–Ovarian epithelial carcinoma cell lines SKOV3, and OVCA429 were obtained from Duke Gynecology/Oncology Bank (Durham, NC). Authentication of cell lines was carried out at the University of Colorado (Denver, CO) sequencing facility. Monkey kidney COS-7 (ATCC® CRL-1651™) cells, mouse

mammary tumor cell line 4T1 (ATCC® CRL2539™), normal Chinese hamster ovarian epithelial cell lines pgsA-745 (ATCC® CRL-2242™) and pgsD-677 (ATCC® CRL-2244™) were obtained from ATCC (Manassas, VA). Epithelial carcinoma cell lines SKOV3, 4T1 and OVCA429 were cultured in RPMI-1640 (ATCC® 30-2001™) containing L-glutamine, 10% FBS, and 100 U of penicillin-streptomycin. COS-7 cells were maintained in DMEM (ATCC® 30-2002™) containing 10% FBS, and 100 U of penicillin-streptomycin. CHO cell lines pgsA-745 and pgsD-677 were cultured in Kaighn's Modification of Ham's F-12 Medium (ATCC® 30-2004™) containing L-glutamine, 10% FBS, and 100 U of penicillin-streptomycin. All cells lines were maintained at 37°C in a humidified incubator at 5% CO_2. Antibodies: phospho-LRP6 (Ser1490) (#2568), LRP6 (#2560), β-catenin (D10A8) XP® Rabbit mAb (#8480), GAPDH Rabbit mAb (#14C10), HA Rabbit mAb (#3724) and Wnt3a (C64F2) Rabbit mAb (#2721) were from Cell Signaling Technology (Danvers, CA). Mouse E-cadherin mAb was purchased from BD Biosciences (#610181) (San Jose, CA). Human betaglycan Antibody (#AF-242-PB) was purchased from R&D Biosystems (Minneapolis, MN) and Actin (#A2228) from Sigma-Aldrich (St. Louis, MO). Mouse HA antibody (#32-6700) from Invitrogen (Carlsbad, CA). Inhibitor SB431542 hydrate (#S4317) was purchased from Sigma-Aldrich. Sodium chlorate ($NaClO_3$) was obtained from Thomas Scientific (Swedesboro, NJ) and sodium sulfate anhydrous (Na_2SO_4) (#S421-500) from ThermoFisher Scientific (Waltham, MA). Heparinase III (#H8891) and Chondroitinase ABC (#C3667) were obtained from Sigma-Aldrich. Recombinant TGF-β1, TGF-β2 and Wnt3a were purchased from R&D Systems.

Plasmid Constructs and Stable Cell Lines–Betaglycan constructs used in this

study have been described previously (9,86,89,129,130). Full length betaglycan consists of betaglycan/TβRIII-HA in pcDNA 3.1(+) as described previously (44,129). pcDNA 3.1(+) betaglycan/TβRIII-ΔGAG construct consists of human betaglycan-HA, with serine-to-alanine point mutations at amino acids 534 and 545 to prevent GAG attachment (44,94,131,132). rTβRIII consists of the rat betaglycan sequence with HA tag in the pcDNA 3.1 vector (89). Adenoviral constructs were used at MOIs between 5-100 particles/cell and infections performed as described previously (9,90,124). shRNA sequences for betaglycan were obtained from Sigma-Aldrich with the following sequences: shRNA33430 (shTβRIII-1): CCGGCCAAGCATGAAGGAACCAAATCTCGAGATTTGGTTCCTTCATGCTTGGT TTTTG and shRNA33432 (shTβRIII-2): CCGGCGTGCTTTATCTCTCCATATTCTCGAGAATATGGAGAGATAAAGCACGT TTTTG in a pLKO.1-puro backbone (betaglycan shRNA construct and non-targeted control). Lentiviral particles were generated at the Center for Targeted Therapeutics Core Facility and the University of South Carolina (Columbia, SC). For betaglycan knockdown, SKOV3 cells were infected with 1X betaglycan shRNA lentivirus. Cells were then selected in the presence of 1 μg ml^{-1} puromycin. Stable cell lines were maintained in 0.5 μg ml^{-1} puromycin.

Wnt3a-HA (#18030) and TβRII-ΔCyto (#14051) plasmids were purchased from Addgene (Cambridge, MA) (133). The soluble human betaglycan construct was a kind gift from Blobe, G. (Duke University, Durham, NC). Conditioned media containing soluble TβRIII/betaglycan was generated by transfecting cells with the indicated expression vectors, and were collected 48 h after transfection in serum-free conditions.

Transient DNA transfections were performed using Lipofectamine 2000 (#11668019) from Life Technologies (Carlsbad, CA) or FuGENE® 6 (#E2691) from Promega (Madison, WI) according to manufacturer's instructions. Cell fractionation kit to analyze β-catenin localization came from Cell Signaling (#9038). Luciferase assay kit (#E1500) came from Promega and M50 Super 8x TOPFlash (134) used to measure luciferase activity was a gift from Randall Moon (Addgene plasmid #12456).

Quantitative Polymerase Chain Reaction (qRT-PCR)–For qRT-PCR, total RNA was isolated from approximately 200K cells using Trizol reagent (Invitrogen). RNA was retro-transcribed using iScript™ Reverse Transcription Supermix (#1708841) and SsoAdvanced Universal SYBR Green Supermix (#1725271) from Bio-Rad (Hercules, CA). qRT-PCR primer sequences used were: RPL13A-F: AGATGGCGGAGGTGCAG, RPL13A-R: GGCCCAGCAGTACCTGTTTA, Betaglycan-F: CGTCAGGAGGCACACACTTA, Betaglycan-R: CACATTTGACAGACAGGGCAAT.

Immunoprecipitation and Western Blotting–Immunoprecipitation and western blotting were performed using standard techniques as described previously (90,93,135). For co-immunoprecipitation in COS-7 cells, betaglycan-expressing cells were transfected with Wnt3a-HA construct indicated and culture media was collected 48 h after transfection in serum-free conditions. betaglycan was then immunoprecipitated by incubating the cell lysates overnight with anti-human betaglycan antibody. The next day, protein G–Sepharose beads were added to the lysates for 2 h at 4°C. The beads were then washed three times with cold phosphate buffer solution (PBS) and resuspended in sample buffer. The amount of betaglycan or Wnt3a bound to the beads was detected by western blot with anti-human betaglycan or Wnt3a antibodies.

Wnt3a-Betaglycan Pull-down Assay–This was performed as described previously (122,136). Briefly, OVCA429 cells were lysed in non-denaturing COIP lysis buffer (50 mM TrisHCl, pH 7.5, 150 mM of NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM DTT, 25 mM NaF, 1 mM Na_3VO_4 and 1X protease inhibitor cocktail [#P8340, Sigma-Aldrich]). betaglycan-HA was then immunoprecipitated by incubating the cell lysates overnight with an anti-human betaglycan antibody. The next day, protein G–Sepharose beads were added to the lysates for 2 h at 4°C. Beads were then washed three times with PBS, and incubated with 20 nM Wnt3a-conditioned media for 2 h at 4°C. After two more washes with PBS, the beads were resuspended in sample buffer and the amount of Wnt3a bound to betaglycan was detected by western blot using anti-Wnt3a and anti-betaglycan antibodies.

Luciferase Assay–Indicated cells were seeded in 24-well plates and were co-transfected with a luciferase reporter vector containing a β-catenin-responsive promoter [to drive luciferase expression (TOPFlash, #12456, Addgene)] and SV40 (*Renilla* internal control vector). One day after transfection and infection, cells were incubated overnight with 50 ng ml^{-1} Wnt3a and then lysed. Luciferase activity (Luciferase Assay System, Promega) was measured by calculating the ratio between luciferase and *Renilla* activities (to normalize for transfection efficiency) and then normalizing values to the untreated sample.

Immunofluorescence and Intensity Analysis–Indicated cells were seeded onto coverslips in 12-well plates at a density of $5x10^4$ cells per well. After infections and treatment with 50 ng ml^{-1} Wnt3a, cells were washed with ice-cold PBS and fixed with 100% methanol for 10 min followed by PBS washes. Cells were permeabilized with

0.1% Triton X-100 in PBS and then blocked with 3% BSA or 0.2% gelatin in PBS for 30 min at room temperature, followed by an overnight incubation at 4°C with a rabbit anti-β-catenin antibody. After extensively washing with PBS, cells were incubated with an Alexa-conjugated secondary antibody (Molecular Probes, Eugene, OR). Cells were mounted in mounting medium and analyzed under an Olympus IX81 motorized inverted microscope (Shinjuku, Tokyo, Japan). Fluorescence intensity for the β-catenin was analyzed using ImageJ 1.50d software (National Institutes of Health, USA) by drawing a fixed line of interest over the membrane and cytoplasm followed by averaging maximum intensities obtained from the plot profile plugin. To estimate the change in β-catenin localization after Wnt treatment, in the presence and absence of betaglycan, the ratio between membrane and cytoplasmic fraction of β-catenin fluorescence was calculated. Statistical significance of the data was analyzed in SigmaPlot version 11software. P-values <0.05 were considered to be statistically significant.

Subcellular Fractionation–Indicated cells were seeded in 12-well plates and infected to express betaglycan. 48 h post infection, cells were treated with 50 ng ml^{-1} Wnt3a for 1 h and then lysed. Subcellular fractionation of β-catenin, cytoplasmic marker GAPDH and plasma membrane marker E-cadherin was carried out using the cell fractionation kit (Cell Signaling) according to manufacturer's instructions.

2.3. Betaglycan suppresses Wnt/β-catenin activity at the level of signal reception.

To investigate the role of betaglycan on signaling by Wnt glycoproteins, which have high affinities for both HSPGs and CSPGs (50,137,138), we expressed betaglycan in the ovarian cancer cell line OVCA429 that we, and others, have established express low levels of betaglycan (Figure 2.1A.i-ii) and (87,124)). Conversely, we reduced

expression of betaglycan by shRNA-mediated knockdown in the ovarian cancer cell line SKOV3, which express higher levels of betaglycan (Figure 2.1A.iii) and (139)). We examined whether betaglycan can affect canonical Wnt signaling as determined by phosphorylation of coreceptor LRP6, one of the first steps initiated by Wnts binding to their signaling coreceptors (140). We find that while Wnt3a robustly phosphorylated LRP6 at serine 1490 (141) in OVCA429 cells (low betaglycan levels), transiently increasing betaglycan expression in OVCA429 cells suppressed Wnt induced LRP6 phosphorylation in a betaglycan dose dependent manner (Figure 2.1B). Total LRP6 levels remained stable in betaglycan-expressing OVCA429 cells when compared to OVCA429 cells with low betaglycan levels (Figure 2.1B). In SKOV3 cells, which express high levels of betaglycan (Figure 2.1A.i), reducing betaglycan expression using shRNA resulted in increased LRP6 phosphorylation when compared to Wnt3a stimulated SKOV3 control cells expressing high endogenous betaglycan (Figure 2.1C-D). To confirm that the effect of shBetaglycan was specific to betaglycan, we utilized shRNA resistant rat betaglycan (9,90) to rescue betaglycan expression and examined Wnt induced LRP6 phosphorylation. We find that rescue of betaglycan expression in shBetaglycan cells (Figure 2.1A.iii) suppressed Wnt induced LRP6 phosphorylation compared to cells containing endogenous betaglycan (Figure 2.1C). Total LRP6 levels were not significantly altered by shRNA to betaglycan or transient expression of rat betaglycan in SKOV3 cells when compared to control cells (Figure 2.1C). Consistently, a second shRNA to betaglycan (shTβRIII-2) also resulted in increased LRP6 phosphorylation when compared to Wnt3a stimulated control cells (Figure 2.1D). These results indicate

that betaglycan may regulate Wnt signaling at the signal reception level by suppressing canonical Wnt signaling.

Activation of the canonical Wnt pathway leads to stabilization and accumulation of cytosolic β-catenin, which then enters the nucleus and regulates Wnt target genes (119). Consistent with reduced LRP6 phosphorylation, Wnt induced β-catenin cytosolic accumulation was significantly reduced in the presence of betaglycan (Figure 2.2A-B).

Upon β-catenin accumulation and stabilization, activation of TCF/LEF-sensitive transcription by β-catenin provides a robust read-out of the Wnt-stimulated canonical pathway (142). To test if betaglycan-mediated changes on LRP6 phosphorylation and β-catenin accumulation translated to downstream effects on TCF/LEF activity, we analyzed the activity of a TCF/LEF-sensitive reporter, which contains multiple β-catenin binding sites (134). We find that Wnt3a significantly increases TCF/LEF reporter activity in OVCA429 cells (Figure 2.2C). Increasing betaglycan expression in these cell lines resulted in a significant suppression of Wnt3a induced activation of the TCF/LEF-reporter compared to control Wnt treated cells (Figure 2.2C). Similar to trends seen in OVCA429 cells, overexpressing betaglycan in SKOV3 cells (betaglycan–high) resulted in suppression of Wnt3a induced TCF/LEF activity compared to control Wnt treated cells (Figure 2.2C). Side-by-side analysis of Wnt3a stimulated TCF/LEF activity in SKOV3 (high betaglycan) and OVCA429 (low betaglycan) cells in the same experiment revealed lower Wnt3a induced TCF/LEF activity in SKOV3 cells, when compared to Wnt3a treated ovarian cancer OVCA429 cells (Figure 2.2D), which we hypothesize is in part due to higher endogenous betaglycan expression in SKOV3 cells (Figure 2.1A, left graph). This hypothesis was confirmed in SKOV3 cells using shRNA to betaglycan

(Figure 2.1A, right graph) that resulted in enhanced Wnt induced TCF/LEF-reporter activity compared to control cells (Figure 2.2E). This increased Wnt signaling in shBetaglycan cells was suppressed upon restoring betaglycan expression using shRNA resistant rat betaglycan (Figure 2.2E), consistent with increased LRP6 activation observed in SKOV3 cells upon knockdown of betaglycan (Figure 2.1C). Regulation of TCF/LEF reporter activity by betaglycan was not restricted to ovarian cancer cells as betaglycan expression also repressed Wnt induced TCF/LEF reporter activity in 4T1 (breast cancer) cells (Figure 2.4D), indicating broad impacts of betaglycan on Wnt signaling regulation.

2.4. TGF-β signaling does not limit betaglycan's ability to suppress Wnt/β-catenin signaling

To begin elucidating the mechanisms by which betaglycan regulates Wnt signaling, we examined whether the presence of TGF-β a high affinity ligand for betaglycan's core domain (143-145), impacted the ability of betaglycan to suppress Wnt signaling. We find that both TGF-β1 and TGF-β2 enhance Wnt induced LRP6 phosphorylation and TCF/LEF activity (Figure 2.3A-B), in OVCA429 cells, but to a lesser extent in SKOV3 cells (high betaglycan) (Figure 2.3C, Lanes 1-4), indicating a cooperative role for TGF-β ligands in Wnt signaling that may be repressed by betaglycan. Treating betaglycan-knockdown SKOV3 cells (shTβRIII) with TGF-β resulted in an enhancement of the Wnt3a-TGF-β cooperativity, compared to control betaglycan-expressing SKOV3 cells treated with Wnt3a and TGF-β (Figure 2.3C, Lanes 5-8). Since TGF-β2 binds the core domain of betaglycan with higher affinity than TGF-β1 (146) and showed the most robust enhancement of Wnt3a induced TCF/LEF activity (Figure 2.3A),

this ligand was chosen to determine TGF-β signaling mediated changes on suppression of Wnt3a induced TCF/LEF activity by betaglycan. We find that Wnt induced TCF/LEF activity, both in the absence and presence of TGF-β2, was dampened by betaglycan expression in OVCA429 cells (Figure 2.3D).

To confirm that betaglycan does not require TGF-β signaling receptors to suppress Wnt signaling, we first utilized SB431542 (inhibitor of TβRI kinase activity) and analyzed Wnt induced TCF/LEF activity in OVCA429 cells. We find that inhibition of TβRI suppressed Wnt signaling independent of betaglycan expression in control cells (Figure 2.3E). However, inhibition of TβRI did not affect betaglycan's ability to suppress Wnt induced TCF/LEF activity in OVCA429 cells when compared to control cells (Figure 2.3E) indicating that Wnt signaling repression by betaglycan is independent of TβRI kinase activity. Several TGF-β independent roles for betaglycan have been reported through its interactions with the Type II TGF-β receptor TβRII (143). However, transient expression of TβRII lacking its cytoplasmic domain (and therefore unable to interact with betaglycan) (TβRII–ΔCyto) (133,143), did not affect betaglycan's ability to suppress Wnt induced TCF/LEF activity when compared to control cells (Figure 2.3F). Similar to Figure 2.3E, removal of TβRII's cytoplasmic domain (TβRII–ΔCyto) in GFP-expressing cells led to a suppression of Wnt induced TCF/LEF activity when compared to control cells (Figure 2.3F). These betaglycan independent observations of TβRII–ΔCyto and SB431542 on TCF/LEF activity may point to autocrine TGF-β-Wnt signaling mechanisms unrelated to betaglycan's ability to suppress Wnt dependent Wnt signaling. Collectively, these data suggest that even in the presence of the high affinity ligand TGF-

β2, the absence of TGF-β signaling and betaglycan-TβRII interaction, betaglycan is still able to suppress Wnt signaling.

2.5. GAG chains of betaglycan regulate Wnt signaling

Wnt glycoproteins have been shown to have a high affinity for GAG chains on transmembrane proteoglycans (50) and the extracellular betaglycan domain contains two sites of HS and CS GAG chains (9,131). To determine if the chains on betaglycan are involved in the suppressive effects on Wnt signaling, we expressed either full-length betaglycan (TβRIII), betaglycan lacking GAG chain modifications (TβRIII-ΔGAG) (30) or control vectors in OVCA429 cells and assessed the levels of phosphorylation of LRP6, cytosolic β-catenin accumulation and TCF/LEF activity induced by exogenous Wnt3a. We find that, unlike full-length betaglycan, TβRIII-ΔGAG failed to suppress LRP6 phosphorylation in OVCA429 cells (Figure 2.4A). Consistently, TβRIII-ΔGAG did not suppress Wnt3a-dependent β-catenin cytoplasmic accumulation compared to full-length betaglycan; instead, β-catenin cytoplasmic accumulation in the presence of TβRIII-ΔGAG resembled cytoplasmic β-catenin levels observed in Wnt3a treated control cells (Figure 2.4B). TβRIII-ΔGAG cells also failed to suppress TCF/LEF activity when compared to full-length betaglycan (Figure 2.4C*)*. The effect of betaglycan's GAG chains on Wnt signaling was not restricted to ovarian cells as TβRIII-ΔGAG also failed to suppress Wnt signaling when compared to full length betaglycan in the murine mammary 4T1 cells (Figure 2.4D).

To test whether the extracellular domain (ECD) of betaglycan was sufficient to suppress Wnt induced signaling, we used two parallel approaches. We treated OVCA429 cells, in the absence and presence of Wnt3a, with either conditioned media (CM) from

cells expressing only betaglycan's ECD (Sol-TβRIII-1) (86,88,144) or CM from cells expressing full-length betaglycan containing soluble betaglycan in the media due to shedding (Sol-TβRIII-2) (30,144) (Figure 2.4E). CM from control vector (GFP) expressing cells was used as control (GFP-CM, Figure 2.4E). These conditions were compared to OVCA429 cells expressing full-length betaglycan in the same experiment (Figure 2.4E). We find that both shed and soluble forms of betaglycan were able to significantly suppress Wnt induced TCF/LEF activity to the same extent as expressing full-length betaglycan (Figure 2.4E). To control for possible artifacts associated with infection of vectors, we also tested media from uninfected cells (Figure 2.4F) and find that infection with GFP did not impact TCF/LEF activity (Figure 2.4F). Taken together, these data confirm that betaglycan's ECD and GAG chains are sufficient at suppressing Wnt induced signaling.

2.6. Betaglycan interacts with Wnt and the balance between HS and CS chains determine betaglycan's ability to regulate Wnt/β-catenin signaling

To determine whether betaglycan binds Wnt3a, we used co-immunoprecipitation of recombinant Wnt3a and betaglycan, a methodology commonly used to study Wnt interactions with its receptors (122,136). We find a Wnt dose dependent interaction between betaglycan and Wnt3a in OVCA429 cells (Figure 2.5A). Consistent with the extracellular domain of betaglycan being sufficient to suppress Wnt signaling (Fig. 4*E*), we find that soluble betaglycan was also able to interact with Wnt3a, as determined by using CM from COS-7 cells expressing full length betaglycan and HA tagged Wnt3a (Figure 2.5B). To determine if the betaglycan–Wnt3a interaction is mediated through betaglycan's GAG chains as suggested by our Wnt signaling assays (Figure 2.4), we

incubated OVCA429 cell lysates with recombinant Wnt3a and performed co-immunoprecipitation in cells expressing full-length betaglycan (TβRIII), TβRIII-ΔGAG or control (Experimental Procedures). We observed immunoprecipitation of Wnt3a and betaglycan that is reduced to background levels in cells expressing TβRIII-ΔGAG (Figure 2.5C). These data indicate that the interaction/binding capacity of TβRIII-ΔGAG is significantly less than full-length betaglycan. These findings are consistent with TβRIII-ΔGAG being unable to inhibit Wnt3a signaling (Figure 2.4B-C).

Since betaglycan represses Wnt signaling and appears to interact with Wnt3a through its GAG chains, we aimed to test whether Wnt signaling regulation by betaglycan's GAG chains are dependent on the sulfation state of betaglycan's GAG chains. We treated betaglycan-expressing OVCA429 cells with sodium chlorate, a competitive inhibitor of ATP-sulfurylase, which results in proteoglycans arriving at the cell surface bearing nonsulfated heparan sulfate or chondroitin sulfate chains (147). We find that non-sulfated GAG chains on betaglycan significantly stimulated Wnt induced TCF/LEF activity (Figure 2.6A). Treatment with sodium sulfate, which overcomes the effects of sodium chlorate and restores sulfation of proteoglycans (147), decreased Wnt induced TCF/LEF activity compared to betaglycan-expressing OVCA429 cells only treated with sodium chlorate (Figure 2.6A). These results demonstrate that the sulfation of betaglycan's GAG chains is required for betaglycan-mediated suppression of Wnt signaling and loss of sulfation results in increased Wnt induced signaling.

Since the GAG chains on betaglycan comprise both HS and CS chains (127), we aimed to isolate the individual effects of the different GAG chains of betaglycan on Wnt signaling. To do this, we first determined whether betaglycan's suppressive role in Wnt

signaling was conserved in parental Chinese Hamster Ovarian (CHO) K1 cells, where betaglycan expresses both HS and CS chains (148). Although CHO cells have a modest response to Wnt stimulation as observed previously (50,149) and by us (Figure 2.6B-C), we observed a significant decrease in Wnt signaling upon betaglycan-expression in CHO K1 cells compared to control cells (Figure 2.6B), consistent with our observations in ovarian and breast cancer cells (Figs. 2.2 and 2.4). To determine the role of betaglycan's CS chains in Wnt signaling, we utilized the CHO cell line derivative pgsD-677, which lack both N-acetylglucosaminyltransferase and glucuronyltransferase activities and are unable to synthesize heparan sulfate, but can produce high amounts of chondroitin sulfate (148). We increased betaglycan expression in pgsD-677 (ΔHS) cells (as described in Experimental Procedures) and examined Wnt induced TCF/LEF activity. Strikingly, we observed a significant increase in Wnt signaling, in betaglycan-expressing pgsD-677 cells compared to control cells (Figure 2.6C). Furthermore, removal of betaglycan's CS chains with chondroitinase (Ch) (Figure 2.6D, *right panel*) reduced Wnt induced TCF/LEF activity in betaglycan-expressing pgsD-677 cells (Figure 2.6D). Since pgsD-677 cells express only CS GAG chains (148), we tested whether CS chains promote Wnt signaling in cells that made both HS and CS GAG chains. Similar to our results in pgsD-677 cells (Figure 2.6D), we find that betaglycan was able to further repress Wnt signaling in OVCA429 cells treated with chondroitinase as compared to control cells (2x repressed, Figure 2.6E). In contrast, heparanase (Hp) treatment of betaglycan-expressing OVCA429 cells resulted in increased TCF/LEF activity compared to heparanase untreated cells (5x increased, Figure 2.6F). These data suggest that HS and CS chains on betaglycan contribute in an opposing fashion, to the availability of Wnt for signaling. Based on these

we propose that the HS chains of betaglycan are responsible for Wnt3a sequestration and subsequent betaglycan–mediated suppression of Wnt3a signaling. In contrast betaglycan's CS chains increase Wnt availability and signaling (Figure 2.6G).

2.7. Discussion and Conclusions

We provide novel evidence for TβRIII/Betaglycan-mediated regulation of canonical Wnt signaling through distinct functions of its HS and CS GAG chains. Our studies demonstrate that the HS chains of betaglycan are responsible for suppression of Wnt3a signaling, most likely via sequestering Wnt, in contrast with the CS chains of betaglycan, which promote Wnt signaling. Based on our findings, we propose that Wnt interactions with the HS chains on betaglycan result in sequestration of Wnt away from LRP6 and Frizzled, which decreases the levels of signaling-productive complexes between the ligand and its receptors. This hypothesis is confirmed upon examining the inability of betaglycan to suppress Wnt signaling upon removal of its GAG chains (Figure 2.4). Mechanistically, our pull-down assays in betaglycan-expressing cells (Figure 2.5) indicate interaction between betaglycan and Wnt glycoproteins, which have a high affinity for polyanionic compounds such as heparin (150), and reveal that the GAG chains significantly increase Wnt-betaglycan interaction to suppress Wnt signaling. Strikingly, betaglycan's CS chains promote Wnt3a signaling in the absence of its HS chains (Figure 2.6B,D). To support this conclusion, chondroitinase treatment in pgsD-677 and OVCA429 cells resulted in a loss of Wnt signaling, thus indicating an exciting new role for the chondroitin chains of betaglycan in stimulating Wnt signaling.

The role of GAG chains in Wnt signal transduction may also depend on the core protein and specific biochemical cues since our data indicate opposing functions for

betaglycan's HS and CS chains in Wnt signaling. To support our hypothesis, it has been shown that exogenous chondroitin sulfate, heparin, and GAGs are unable to stimulate Wnt3a signaling while endogenous CSPGs promote Wnt signaling in mouse L cell fibroblasts, suggesting that the core proteins of CSPGs may be involved in regulating Wnt3a activity (137). We speculate that localization, sulfation and/or chain length of GAG chains attached to core proteins could contribute to differences in ligand availability and signaling.

Studies have also shown that cell context can determine the role proteoglycans and GAG chains play in cancer progression. For instance, enzymatic elimination of chondroitin sulfate molecules in primary breast tumors, for example, increases lung metastases in mice (151) while digestion of cell surface CS on lung cancer cells injected into tail veins leads to a reduction in the number of tumor cells able to populate and metastasize (152). These results suggest that CS molecules may have opposing roles during cancer progression: an anti-metastatic function in primary tumor tissue and a pro-metastatic role during extravasation (circulating cancer cell interaction with endothelial cells) (153). Other proteoglycans have also been shown to function as either tumor promoters or suppressors depending on the protein core, GAG chains attached, associated molecules, proteoglycan localization and tumor type (154). Perlecan, for example, can both promote tumor invasiveness (155) and inhibit angiogenesis (156) while glypicans and syndecans may promote local cancer cell growth and metastatic potential in some cancer tissues (138,157), but inhibit tissue growth, invasion and metastasis in others (158,159). Together, these data show a requirement for the proteoglycan's core domain and cellular environment in deciding GAG chain function.

In addition to the contributions made by the proteoglycan's core domain and environment, the sulfation state of the proteoglycan also plays a major role in its ability to regulate signaling pathways. Upon treatment of our betaglycan-expressing OVCA429 cells with sodium chlorate, an ATP-sulfurylase competitive inhibitor that causes proteoglycans to arrive at the cell surface bearing nonsulfated HS or CS chains (147), we find betaglycan unable to repress Wnt signaling, indicating that the sulfation of betaglycan's GAG chains is required for proper Wnt signal regulation by betaglycan (Fig. 6*A*), consistent with previous reports for Glypican-1 (50). Studies in *Drosophila* have also shown that, upon treatment of *Drosophila* cells with sodium chlorate or in the absence of an HS N-deacetylase/N-sulfotransferase, cells are completely deficient in HS chain sulfation and Wingless [Wg] signaling is disrupted (160-163). HS chain sulfation plays a vital role in regulating FGF signaling as well. Consistently, the HS chains of betaglycan can also regulate FGF signaling and play a critical role in tumor progression (9).

Previous reports indicate that FGF signal transduction is dependent on sulfation of the 2-O and 6-O positions on HS chains, which control FGF1 binding to heparin and FGF1-dependent dimerization and activation of the FGFR1 receptor, respectively (164-166). In articular cartilage, studies reveal a Wnt signaling promoter role for CS chains that is dependent on the sulfation of the CS chain (167). Taken together, these studies, combined with our data, suggest that sulfation plays a significant role in growth factor signaling regulation by GAG chains on proteoglycans.

It is possible that different expression levels of β1,4-N-acetylgalactosaminyltransferase-I (β4GalNAcT-I) and/or α1,4-N-

acetylglucosaminyltransferase-I (α4GlcNAcT-I), which initiate the synthesis of CS or HS chains, respectively, may also contribute to betaglycan's proteoglycan state and subsequent effects on Wnt signaling. Moreover, within a single core protein, Ser-Gly residues in a hydrophobic pocket might signal heparan sulfate attachment, while Ser-Gly residues in an exposed hydrophilic environment might signal chondroitin sulfate attachment. These different local environments could achieve selectivity by modulating the activity of β4GalNAcT-I and α4GlcNAcT-I (168). Other biochemical cues may include the location of N-linked glycosylation sites (Asn-Phe-Ser) as described for Syndecan-1 (34). Attachment of an N-linked sugar at a GAG chain attachment site would likely prevent subsequent recognition by the xylosyltransferase and GAG chain attachment to betaglycan's core protein.

The precise mechanism by which CS chains of betaglycan increase Wnt availability remains to be determined. Future studies into the biochemical cues involved in determining the proteoglycan state of HSPGs, such as betaglycan, as well as betaglycan's role in regulating Wnt signaling will help shed light on Wnt signaling regulation and increase our understanding of the diverse roles proteoglycans like betaglycan play in signaling and disease.

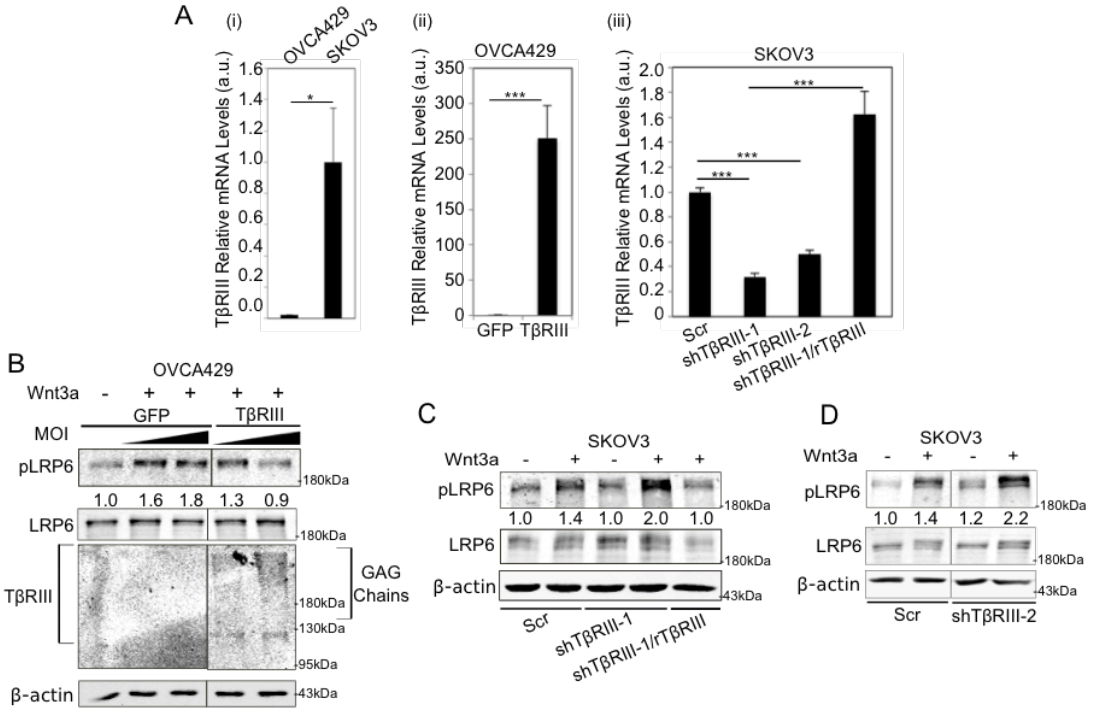

Figure 2.1. TβRIII suppresses Wnt/β-catenin activity at the level of signal reception. (A) TβRIII mRNA expression by qRT-PCR analysis to detect (i) endogenous TβRIII levels in OVCA429 and SKOV3 cells, (ii) overexpression of TβRIII in OVCA429 cells as indicated and (iii) expression in SKOV3 cells of indicated shRNA and rescue conditions generated as described in Experimental Procedures. Ct values normalized in graph (i) to endogenous TβRIII levels in SKOV3 cells (lane 2), in graph (ii) to GFP (lane 1) and in graph (iii) to Scr TβRIII levels (lane 1). Quantitations represent the average of two independent biological trials each conducted in triplicate. (B) OVCA429 cells transiently expressing increasing doses of TβRIII (5 & 10 MOI of TβRIII expressing adenoviral constructs) or control (GFP) were stimulated with 50 ng ml^{-1} Wnt3a for 1 h followed by immunoblotting of lysates for phospho-LRP6 (Ser^{1490}) (pLRP6), LRP6, TβRIII and β-actin. (C) SKOV3 cells transiently expressing shRNA to TβRIII (shTβRIII-1) or Scrambled (Scr) control (Experimental Procedures) and transiently transfected with rat TβRIII (rTβRIII) or control vector (pcDNA 3.1) for 24 h (for rescue of TβRIII expression as seen in 2.1A, right panel), and then stimulated with 50 ng ml^{-1} Wnt3a for 1 h followed by immunoblotting of lysates for phospho-LRP6 (Ser^{1490}) (pLRP6), LRP6 and β-actin. (D) SKOV3 cells transiently expressing a second, independent shRNA to TβRIII (shTβRIII) or Scrambled control (Experimental Procedures) were stimulated with 50 ng ml^{-1} Wnt3a for 1 h followed by immunoblotting of lysates for phospho-LRP6 (Ser^{1490}) (pLRP6), LRP6 and β-actin. (B-D) Quantitations represent pLRP6: LRP6 ratios and are normalized to the untreated sample. All sub-figures (A-D) represent at least two independent biological trials.

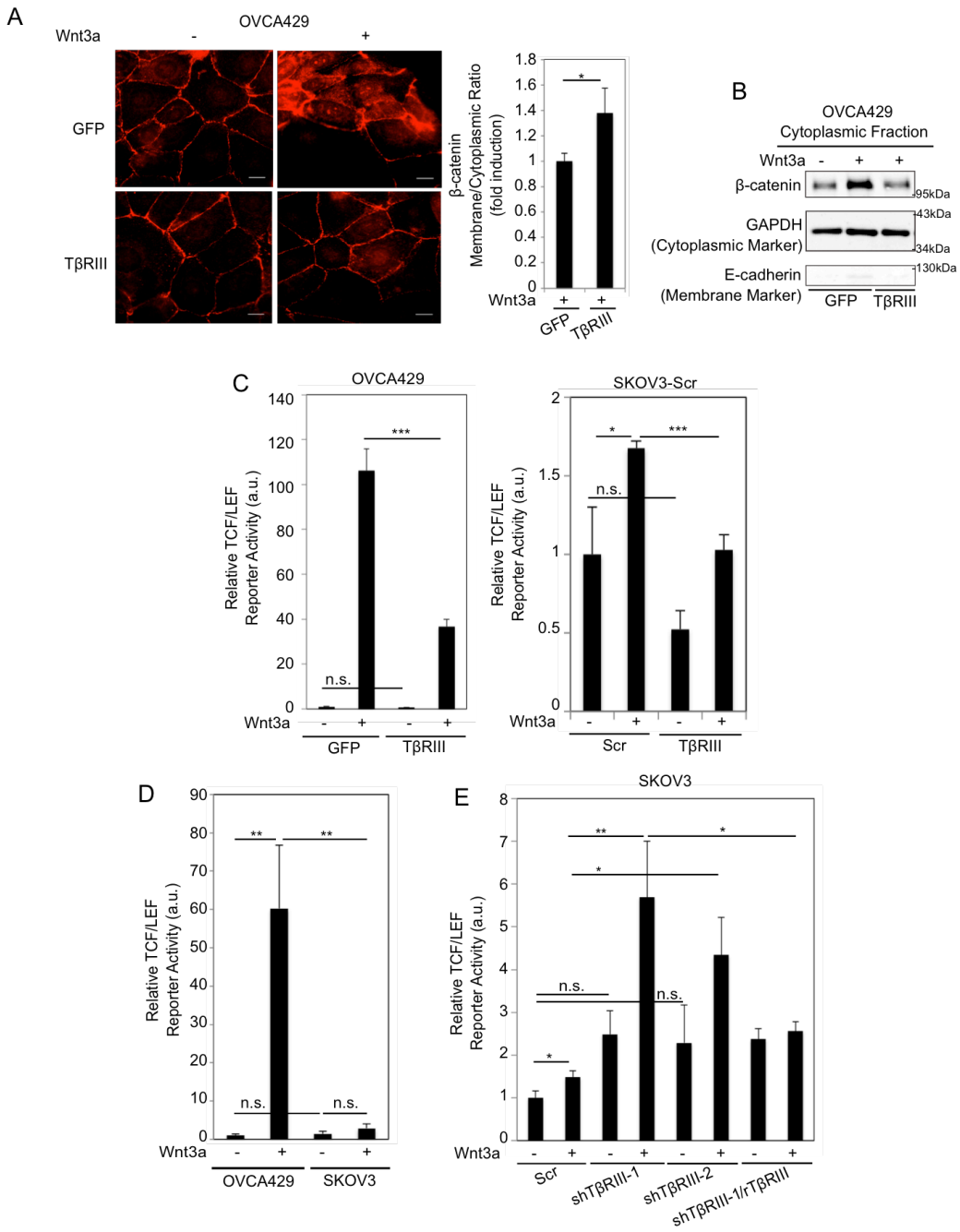

Figure 2.2. TβRIII suppresses Wnt induced β-catenin cytoplasmic accumulation and transcriptional activity. (A) OVCA429 cells transiently expressing control (GFP) or TβRIII were stimulated with 50 ng ml^{-1} Wnt3a for 1 h and immunostained for β-catenin (red). Scale bars: 20 μm. Graph represents quantitation of β-catenin fluorescence at the membrane versus cytoplasm (Experimental Procedures). N ≥ 30 cells/condition. Values normalized to control GFP. Figure represents at least two independent biological trials. (B) Cytoplasmic fractions obtained after subcellular fractionation (Experimental Procedures) of OVCA429 cells transiently expressing TβRIII or GFP stimulated with 50 ng ml^{-1} Wnt3a for 1 h followed by immunoblotting of lysates for β-catenin, GAPDH (positive cytoplasmic marker) and E-cadherin (negative cytoplasmic marker). Figure

represents at least two independent biological trials. (C-E) Indicated cells expressing either TβRIII, shTβRIII, or shTβRIII with rTβRIII, as in Fig. 2.1*B-C*, were transfected with a Wnt-responsive luciferase reporter and SV40 control vector and left untreated or treated with 50 ng ml^{-1} Wnt3a for 24 h. Luciferase activity was then measured as described in Experimental Procedures. All values normalized to the untreated sample and represent the average of two independent biological trials each conducted in duplicate. Data analyzed using two-tailed Student's t-test and represent the mean±s.e.m.

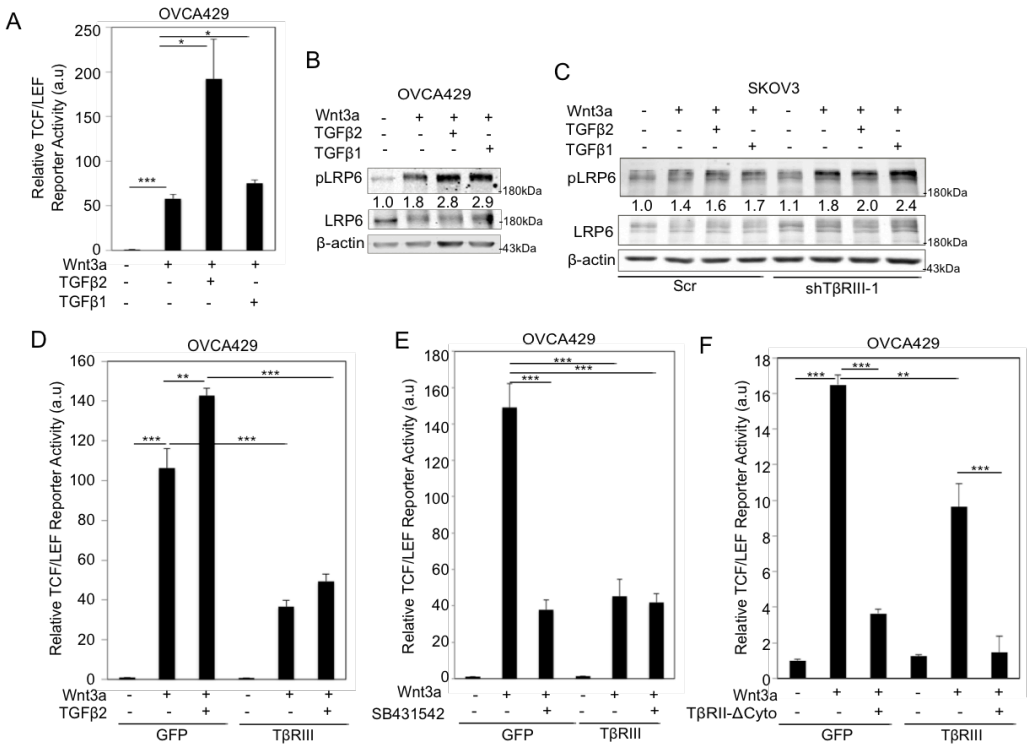

Figure 2.3. TGF-β signaling does not limit TβRIII's ability to suppress Wnt/β-catenin signaling. (A) OVCA429 cells transfected with a Wnt-responsive luciferase reporter (500 ng) and a SV40 control vector were either untreated or stimulated with 50 ng ml^{-1} Wnt3a and 400 pM TGF-β1 or TGF-β2 for 24 h. Luciferase activity was then measured as described in Experimental Procedures. (B) OVCA429 cells were either untreated or stimulated with 50 ng ml^{-1} Wnt3a and 400 pM TGF-β1 and TGF-β2 as indicated for 1 h. Cells were lysed and levels of phospho-LRP6 (Ser^{1490}) (pLRP6) and β-actin were assessed by immunoblotting. Quantitations represent pLRP6: LRP6 ratios and are normalized to the untreated sample. (C) SKOV3 cells stably expressing control or TβRIII shRNA-1 were either untreated or stimulated with 50 ng ml^{-1} Wnt3a and 400 pM TGF-β1 or TGF-β2 for 1 h. Cells were assessed as in 1*B*. Quantitations represent pLRP6:LRP6 ratios and are normalized to the untreated sample. (D-F) OVCA429 cells transiently expressing either control (GFP) or TβRIII were transfected with a Wnt-responsive luciferase reporter and a SV40 control vector and incubated with (D) 50 ng ml^{-1} Wnt3a in the presence or absence of 400 pM TGF-β2, (E) 50 ng ml^{-1} Wnt3a and 5 μM SB431542 or (F) 50 ng ml^{-1} Wnt3a and a dominant negative form of the Type II TGF-β receptor (TβRII-ΔCyto) or pcDNA 3.1 control vector for 24 h. Luciferase activity was measured as described in Experimental Procedures. Luciferase data are representative of at least two independent biological trials each conducted in duplicate. Data analyzed using two-tailed Student's t-test and represent the mean±s.e.m. Western analysis data represent at least two independent biological trials. All values normalized to the untreated sample.

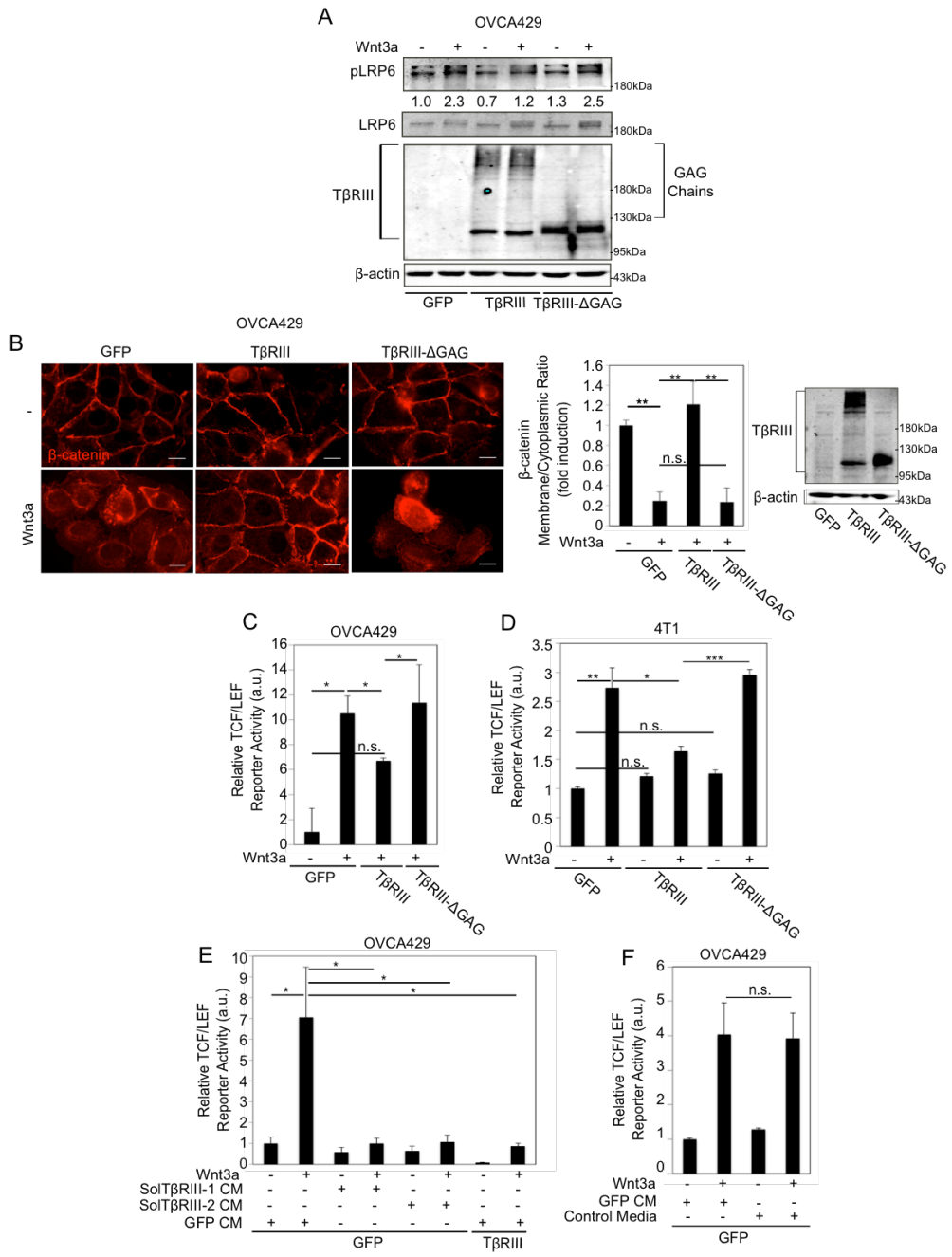

Figure 2.4. GAG chains of TβRIII suppress Wnt signaling. (A) OVCA429 cells transiently expressing full-length TβRIII, TβRIII-ΔGAG or GFP (control) were stimulated with 50 ng ml^{-1} Wnt3a. Cells were then lysed after 1 h and phospho-LRP6 (Ser^{1490}) (pLRP6), LRP6 and β-actin levels assessed by immunoblotting. Quantitations represent pLRP6:LRP6 ratios and are normalized to the untreated sample. (B) Indicated OVCA429 cells were stimulated with 50 ng ml^{-1} Wnt3a for 1 h and immunostained for β-catenin (red). Scale bars: 20 μm. Graph represents quantitation of β-catenin fluorescence at the membrane versus cytoplasm (Experimental Procedures). N ≥ 30 cells/condition. Western analysis shows TβRIII and β-actin levels. Data analyzed using ANOVA followed by post hoc Shapiro-Wilk test and represent the mean±s.e.m. All values

normalized to the untreated sample. (C-F) Indicated cells were stimulated with either (C-D) 50 ng ml^{-1} Wnt3a alone, (E) 50 ng ml^{-1} Wnt3a in the presence of either conditioned media (CM) from cells expressing only TβRIII-ECD (Sol-TβRIII-1), CM from cells expressing full-length TβRIII (Sol-TβRIII-2), or CM media from control GFP expressing cells for 24 h or (F) 50 ng ml^{-1} Wnt3a in the presence of control media from untransfected cells or from cells transiently expressing GFP. Cells were lysed after 24 h and luciferase activity measured in 1% serum as described in Experimental Procedures. All values normalized to the untreated sample. All luciferase data (C-F) are representative of at least two independent biological trials each conducted in duplicate. Data analyzed using two-tailed Student's t-test and represent the mean±s.e.m of a biological replicate. All values normalized to the untreated sample. All figures represent at least two independent biological trials.

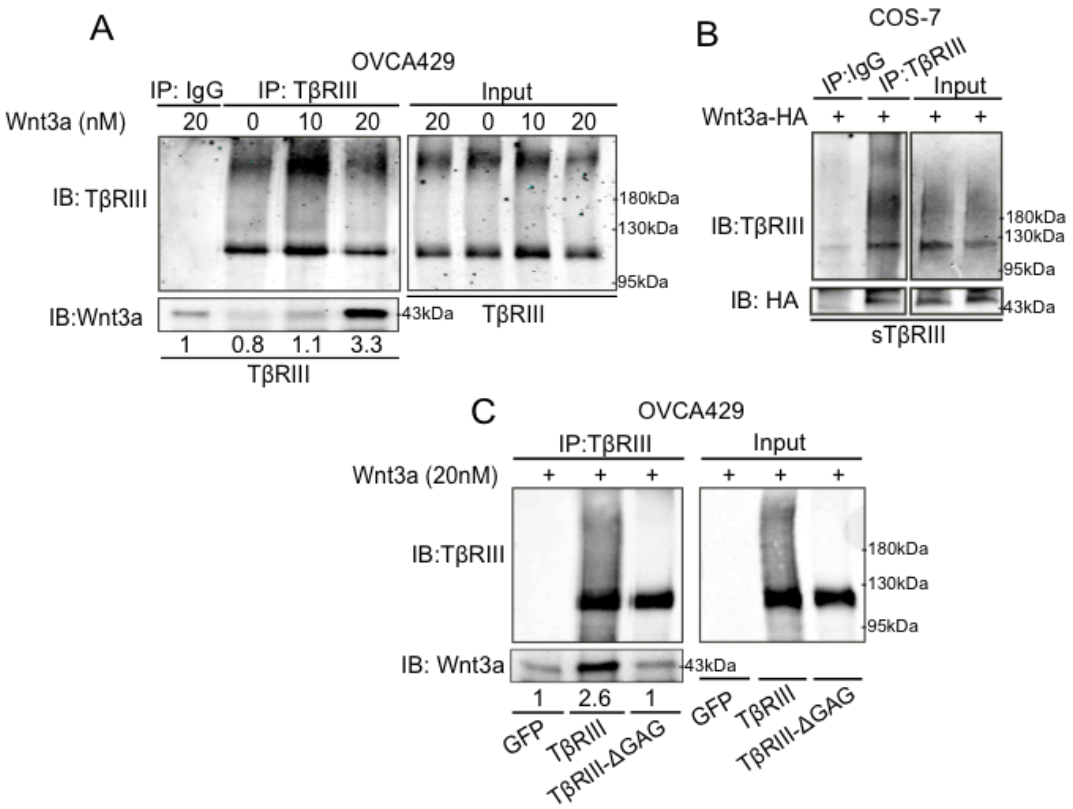

Figure 2.5. TβRIII interacts with Wnt through its GAG chains. (A) OVCA429 cells transiently expressing full length TβRIII were immunoprecipitated and analyzed for Wnt3a-TβRIII interactions using the Wnt pull-down assay described in Experimental Procedures and previously in (122). All values normalized to the IgG sample. (B) Conditioned media from COS-7 cells transiently expressing full length TβRIII and Wnt3a-HA was immunoprecipitated using anti-TβRIII and immunoblotted using anti-HA and anti-TβRIII to analyze Wnt3a-TβRIII interactions as described in Experimental Procedures and previously in (90,93,135). (C) OVCA429 cells transiently expressing GFP (control), full length TβRIII or TβRIII-ΔGAG were immunoprecipitated using anti-TβRIII and immunoblotted using anti-Wnt3a and anti-TβRIII to analyze Wnt3a-TβRIII interactions using the Wnt pull-down assay described in Experimental Procedures and previously in (122) All values normalized to the GFP sample and all figures represent at least two independent biological trials.

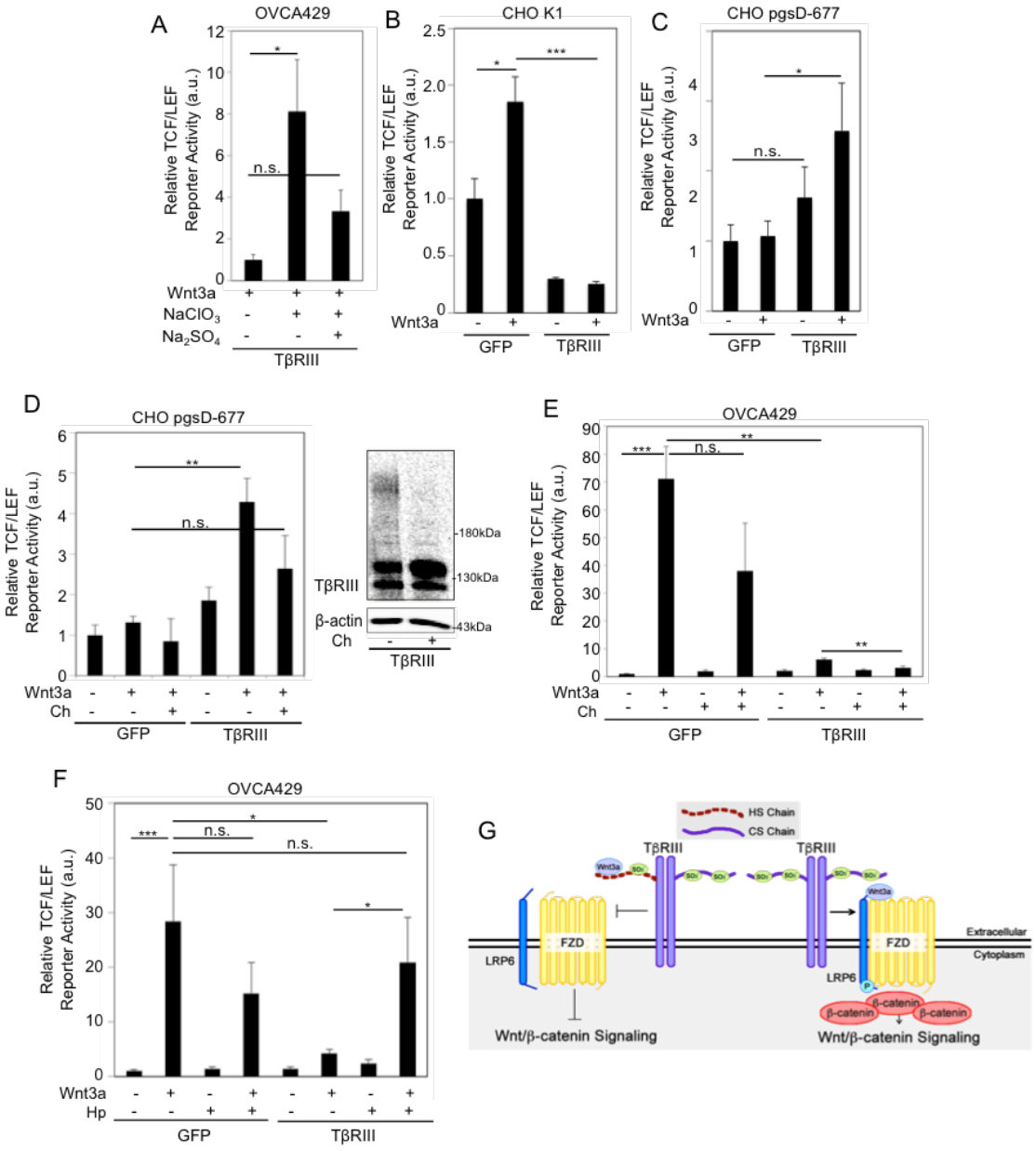

Figure 2.6. The balance between sulfated heparan and chondroitin chains on TβRIII determines TβRIII's ability to regulate Wnt/β-catenin signaling. (A) OVCA429 cells transiently expressing full length TβRIII, transfected with a Wnt-responsive luciferase reporter and a SV40 control vector were pre-treated with 50 mM $NaClO_3$ with or without 10 mM Na_2SO_4 as indicated for 2 h. Cells were then stimulated with 50 ng ml^{-1} Wnt3a and luciferase activity was measured as described in Experimental Procedures. All values normalized to the Wnt treated sample. (B-F) CHO K1, pgsD-677 or OVCA429 cells expressing TβRIII or GFP were transfected with a Wnt-responsive luciferase reporter and a SV40 control vector and pre-treated with (D-E) 100 mU ml^{-1} chondroitinase (Ch) or (F) 20 mU ml^{-1} heparanase (Hp) for 2 h before overnight incubation with 50 ng ml^{-1} Wnt3a. Luciferase activity was measured as described in Experimental Procedures. All values normalized to the untreated sample. Western analysis in (D) shows TβRIII expression in

pgsD-677 cells after CS chain removal using 100 mU ml^{-1} chondroitinase. All data represent at least two independent biological trials. Data analyzed using two-tailed Student's t-test and represent the mean±s.e.m. (G) Model of canonical Wnt/β-catenin signaling regulation by TβRIII.

CHAPTER 3

GENERATION AND CHARACTERIZATION OF MODIFIED BETAGLYCAN AND SDC1 MUTANTS USING SITE DIRECTED MUTAGENESIS

3.1. Introduction

Site-directed mutagenesis (SDM), a priceless tool used to modify DNA sequences for molecular biological studies and genetic engineering, is commonly used to study protein structure-function relationships with most mutagenesis methods based on PCR. To support the enzymatic digestion studies performed on full-length betaglycan (see Figure 2.6) and define new roles for SDC1's individual GAG chains in cell signaling and cancer biology, we employed the simplest and most broadly applicable SDM protocol, the QuikChange Site-Directed Mutagenesis System (QCM) (Stratagene, La Jolla, CA), and synthesized betaglycan and SDC1 mutants containing either CS or HS chains exclusively.

The core domain of rat betaglycan contains six serine-glycine sequences of which only Ser^{535}-Gly and Ser^{546}-Gly are surrounded by the required acidic amino acids and tryptophan residue (Trp^{537}) needed for GAG chain attachment (30,169-171). In humans, sequence alignment places these serines at the 534 and 545 amino acid positions ((9) and Figure 3.1). Previously, using SDM, rat betaglycan containing serine-alanine mutations at serine 545 and serine 546 were expressed in monkey kidney fibroblast-like COS-1 cells, affinity labeled with $^{I\text{-}125}$TGFβ1, cross-linked with disuccinimidyl suberate (DSS) and labeled receptors were revealed by autoradiography. Mutation of either serine produced betaglycan forms of faster electrophoretic migration, causing an increase in GAG-less core protein expression (30). Double mutation of both serines completely abolished the addition of GAG chains while heparitinase and chondroitinase enzymatic digestions of these mutated betaglycan forms identified both HS (predominately) and CS chains at serine 535 and CS chains exclusively at serine 546 (30,170).

In the case of SDC1, similar SDM of Ser-Gly sites followed by enzymatic

digestion via heparitinase and chondroitinase revealed three N-terminal cluster sites (DGSGD and FSGSGTG) for HS chain attachment ($Ser^{37,45,47}$) and two C-terminal cluster sites (EGSGE and ETSGE) for CS chain attachment ($Ser^{207, 217}$) in mice ((34,170) and Figure 3.2). Sequence alignment indicates human SDC1 contains two HS chain attachment sites ($S^{45, 47}$) and three sites for CS chain attachment ($S^{37,206,216}$). Analysis of betaglycan and SDC1's HS and CS GAG chain composition and features are elaborated on in Section 1.1.ii.

Using QCM, I introduced serine-to-alanine point mutations in betaglycan and SDC1, in a single PCR with one pair of complementary primers containing our mutation of interest, and generated the following betaglycan and SDC1 constructs: human betaglycan mutated at serine 545, which I have characterized to contain predominately HS chains (pcDNA3.1(+)-hBetaglycan-S545A-HA/TβRIII-S545A) (8.6kb), human betaglycan mutated at serine 534, which I have characterized to contain CS chains exclusively (pcDNA3.1(+)-hBetaglycan-S534A-HA/TβRIII-S534A) (8.6kb) and murine SDC1 mutated at serine 207 and 217 (pCMV2-mSDC1-ΔCS-Flag) (6.99kb). Characterization of this SDC1 mutant, to confirm the exclusive attachment of HS chains as described in (34,170), will be done in the future.

After sequencing all new DMTP constructs (to verify successful SDM), I expressed these new DMTP forms in different cell types, enzymatically digested the GAG chains and then compared the results to human full-length betaglycan (pcDNA 3.1(+)-hBetaglycan-FL-HA/TβRIII-FL-HA) (8.6kb), human betaglycan without GAG chains (pcDNA3.1(+)-hBetaglycan-ΔGAG-HA/TβRIII-ΔGAG-HA) (8.6kb), murine full-length SDC1 (pCMV2-mSDC1-FL-Flag) (6.99kb), murine SDC1 with no HS chains

(pcDNA3-mSDC1-ΔHS) (6.99kb) and/or a GAG-less murine SDC1 (pcDNA3-mSDC1-ΔGAG-Flag) (6.99kb) to confirm previously established (30,34,170) GAG chain attachment sites on betaglycan and SDC1.

3.2. Experimental Procedures

SDM Plasmid Construct Templates–Human betaglycan constructs used in this study have been described previously (9,86,89,129,130). Full-length betaglycan consists of human betaglycan-HA in pcDNA 3.1(+) (pcDNA3.1-hBetaglycan-HA) as described in (44,129,130). The pcDNA 3.1(+)-hBetaglycan-ΔGAG-HA construct consists of human betaglycan-HA, with serine-to-alanine point mutations at amino acids 534 and 545 to prevent GAG attachment (44,94,131,132). Full-length murine SDC1 construct, pCMV2-mSDC1-Flag (#MG50641-M-F), was purchased Sino Biological (Beijing, China). Mouse pcDNA3-mSDC1-ΔGAG-Flag (69) contains serine-to-alanine point mutations at amino acids 37, 43, 45, 47, 207 and 217 to prevent GAG attachment while murine pcDNA3-mSDC1-ΔHS (53) contains serine-to-alanine point mutations at 37, 45 and 47 to eliminate HS chain attachment. These SDC1 constructs were kind gifts from Sanderson, R. (University of Arkansas, Little Rock, Arkansas). Transient DNA transfections of these constructs were performed using Lipofectamine 3000 (#L3000008) from ThermoFisher Scientific (Waltham, MA) or FuGENE® 6 (#E2691) from Promega (Madison, WI) according to manufacturer's instructions.

Site Directed Mutagenesis-All primers designed to introduce the SDM were synthesized and purified by Integrated DNA Technologies (Coralville, IA). The PCR amplification was carried out with either QuikChange II XL Site-Directed Mutagenesis (Cat#200521, Agilent Technologies, Santa Clara, CA) (for pcDNA3.1(+)-hBetaglycan-

S534A-HA), Q5® Site-Directed Mutagenesis Kit (Cat#E0554S, New England BioLabs, Ipswich, MA) (for pcDNA3.1(+)-hBetaglycan-S545A-HA) or QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Cat#210515, Agilent Technologies) (for pCMV2-mSDC1-ΔCS-Flag) per manufacturer's instructions.

Stable Cell Line Generation-To generate stable cells lines, each betaglycan construct was first cloned into an intermediate TOPO cloning vector and then into a pHIV-dTomato lentiviral backbone (Plasmid #21374, Addgene, Cambridge, MA) by the Center for Targeted Therapeutics Core Facility and the University of South Carolina (Columbia, SC). Lentiviral particles were then generated at the Center for Targeted Therapeutics Core Facility. Ovarian cancer line HEYA8 (gift from Murphy, S., University of South Carolina, Columbia, SC) monkey fibroblast kidney cell line COS-7 (#CRL-1651, ATCC®) and human embryonic kidney cell line HEK293 (#CRL-1573, ATCC®) were infected with 1X betaglycan mutant lentivirus for 72 hr. HEYA8 cells were cultured in RPMI-1640 (ATCC® 30-2001™) containing L-glutamine, 10% FBS, and 100 U of penicillin-streptomycin. COS-7 and HEK293 cells were maintained in DMEM (ATCC® 30-2002™) containing 10% FBS, and 100 U of penicillin-streptomycin. Cells were then sorted using flow cytometry at a cell density of 4 x 10^6/ml at the Center for Targeted Therapeutics Core Facility. Cell fractionation kit to analyze betaglycan localization came from Cell Signaling (#9038). For all primers, mutagenized positions are denoted in boldface and underlined. Reverse primers with a completely complementary role are marked with an asterisk.

Quantitative Polymerase Chain Reaction (qRT-PCR)–For qRT-PCR, total RNA was isolated from approximately 200K cells using Trizol reagent (Invitrogen). RNA was

retro-transcribed using iScript™ Reverse Transcription Supermix (#1708841) and SsoAdvanced Universal SYBR Green Supermix (#1725271) from Bio-Rad (Hercules, CA). qRT-PCR primer sequences used were: RPL13A-F: AGATGGCGGAGGTGCAG, RPL13A-R: GGCCCAGCAGTACCTGTTTA, Betaglycan-F: CGTCAGGAGGCACACACTTA, Betaglycan-R: CACATTTGACAGACAGGGCAAT. Ovarian cancer lines not previously described include OVCAR5, OVCAR8, M41, OVCA420 and were obtained from the Duke Gynecology/Oncology Bank (Durham, NC). Authentication of cell lines was carried out at the University of Colorado (Denver, CO) sequencing facility. Cells were cultured in RPMI-1640 (ATCC® 30-2001™) containing L-glutamine, 10% FBS, and 100 U of penicillin-streptomycin.

Immunoprecipitation and Western Blotting–Immunoprecipitation and western blotting were performed using standard techniques as described previously (90,93,135). Recombinant *P. heparinus* Heparinase III Protein (#6145-GH-010) was obtained from R&D Systems while Chondroitinase ABC (#C3667) was purchased from Sigma-Aldrich. For co-immunoprecipitation in betaglycan-expressing HEK293 cells, betaglycan was immunoprecipitated by incubating the cell lysates overnight with 6 μg of an anti-chicken Heparan Sulfate Proteoglycan antibody (#33 or 33-2-s, Developmental Studies Hybridoma Bank, Iowa City, IA). The next day, protein G–Sepharose beads were added to the lysates for 2 h at 4°C. The beads were then washed three times with cold phosphate buffer solution (PBS) and resuspended in sample buffer. The amount of betaglycan bound to the beads was detected by western blot with an anti-human betaglycan antibody (#AF-242-PB, R&D Biosystems).

3.3. Sequence and generation of DMTP constructs

For generation of pcDNA3.1(+)-hBetaglycan-S534A-HA, 50 μl PCR reaction was carried out with 100 ng template (pcDNA3.1(+)-hBetaglycan-ΔGAG-HA), 250 ng primer pair, 1 μl of Agilent dNTPs, 3 μl QuikSolution and 2.5 U *PfuUltra* HF DNA polymerase. The extension reaction was initiated by pre-heating the reaction mixture to 95^{o}C for 1 min; 18 cycles of 95^{o}C for 50 sec, 60^{o}C for 50 sec and 68^{o}C for 1 min/kb of plasmid length; followed by incubation at 68^{o}C for 7 min.

To generate pcDNA3.1(+)-hBetaglycan-S545A-HA, 20 μl PCR reaction was carried out with 500 ng template (pcDNA3.1(+)-hBetaglycan-FL-HA), 2 μl 10 μM primer pair, 1 μl 10 mM dNTPs, 4 μl Q5 Buffer (Cat#E0554S, New England BioLabs) and 0.25 μl Q5 DNA polymerase (Cat#E0554S, New England BioLabs). The extension reaction was initiated by pre-heating the reaction mixture to 98^{o}C for 3 min; 25 cycles of 98^{o}C for 1 min, 65^{o}C, 70^{o}C and 72^{o}C for 30 sec and 72^{o}C for 2.5 min/kb; followed by incubation at 72^{o}C for 2 min.

pCMV2-mSDC1-ΔCS-Flag, a murine SDC1 mutant with serine-to-alanine point mutations at amino acids 207 and 217 to prevent CS attachment, was generated using a 25 μl PCR reaction containing 100 ng template (pCMV2-mSDC1-FL-Flag), 0.75 μl QuikSolution, 200 ng primer pair, 1 μl of Agilent dNTPs and 1 μl QuikChange Multi enzyme blend. The extension reaction was initiated by pre-heating the reaction mixture to 95^{o}C for 1 min; 30 cycles of 95^{o}C for 1 min, 55^{o}C for 1 min and 65^{o}C for 2 min/kb of plasmid length according to the length of template; followed by incubation on ice for 2 min to cool the reaction to ≤37°C.

PCR-amplified products were evaluated by agarose gel electrophoresis, purified by QIAquick PCR purification kit (Cat#28104, Qiagen, Germany) and further treated with restriction enzyme DpnI for 2 hr at 37^{o}C. A 3 μl aliquot of each PCR product was transformed into XL10-Gold Ultracompetent cells and inoculated on Luria–Bertani (LB) plate containing 100 mg/ml ampicillin. A total of 10-12 colonies were selected and their plasmids were isolated by mini-prep. All plasmids were then sequenced by Eton Bioscience, Inc. (Research Triangle Park, NC) to identify positive mutants.

3.4. Characterization of human betaglycan-S534A-HA and -S545A-HA

To determine individual roles for betaglycan's GAG chains in cell signaling and cancer, I used SDM to insert a serine-to-alanine point mutation at serine 534 in full-length betaglycan or an alanine-to-serine point mutation at alanine 545 in hBetaglycan-ΔGAG-HA to generate betaglycan containing either CS chains exclusively (hBetaglycan-S534A-HA/TβRIII-S534A) or betaglycan containing predominately HS chains (hBetaglycan-S545A-HA/TβRIII-S545A) respectively (Figures 3.3 and 3.5A).

PCR amplification of both hBetaglycan-S534A-HA and hBetaglycan-S545A-HA yielded satisfactory quantities of amplification products (Figure 3.5B) for cloning into an intermediate TOPO cloning vector. From the TOPO vector, these betaglycan mutants were cloned into a pHIV-dTomato lentiviral backbone for stable cell line generation and transient betaglycan expression analysis.

To evaluate expression of the mutants, I transiently expressed betaglycan constructs pHIV-dTomato-hBetaglycan-FL-HA, -S534A-HA, -S545A-HA and -ΔGAG-HA in both COS-7 and HEK293 cells (Figure 3.5C) and observed betaglycan core protein expression for all mutants with varying degrees of GAG chain expression. I also

performed betaglycan immunoprecipitation using a HSPG antibody in betaglycan-expressing HEK293 cells and confirmed that betaglycan in HEK293 cells can express HS chains (Figure 3.5D). A similar immunoprecipitation in betaglycan expressing HEK293 cells using a chondroitin sulfate proteoglycan antibody is warranted, as it would reveal whether CS chains attach to betaglycan in this cell line.

To confirm that pHIV-dTomato-hBetaglycan-S534A-HA (TβRIII-S534A) expresses CS chains exclusively and to identify the GAG chains attached at serine 534 on pHIV-dTomato-hBetaglycan-S545A-HA (TβRIII-S545A) in cell lines, I first stably expressed these modified betaglycan constructs in COS-7 and HEYA8 cells (Figure 3.5F-G). HEYA8 cells were identified as a low TβRIII-expressing ovarian cancer cell line based on qRT-PCR analysis of seven epithelial cell lines (Figure 3.5E). I then enzymatically digested the CS and HS chains on betaglycan using chondroitinase and heparitinase, respectively. In both COS-7 and HEYA8 cells, I find that treatment of hBetaglycan-S534A-HA with chondroitinase removed CS chains from betaglycan and increased betaglycan's GAG-less core protein pools, indicating this modified form of betaglycan expresses only CS chains (Figure 3.5F). These results are thus consistent with prior reports on betaglycan's GAG chain attachment sites (9,30,170). When hBetaglycan-S545A-HA (TβRIII-S534A) expressing COS-7 and HEYA8 cells were treated with heparitinase alone, the majority of betaglycan's GAG chains were removed, resulting in a large pool of betaglycan core protein with no GAG chains. When these same cells were treated with both chondroitinase and heparitinase, no reduction in GAG chain levels on hBetaglycan-S545A-HA was observed in COS-7 and only a slight reduction in GAG chain expression on HEYA8 hBetaglycan-S545A-HA was observed, indicating that

hBetaglycan-S545A-HA/TβRIII-S545A exclusively expresses HS chains in COS-7 cells and likely in HEYA8 cells (Figure 3.5G).

To support my previously outlined (Figure 2.6) roles for betaglycan's individual chains on Wnt signaling, I analyzed phospho-LRP6 and LRP6 basal levels in HEYA8 cells lines stably expressing modified betaglycan forms. Compared to HEYA8 cells expressing full-length betaglycan, I observed an increase in both phosphorylated and total LRP6 levels in hBetaglycan-ΔGAG-expressing HEYA8 cells (TβRIII-ΔGAG)(Figure 3.5H), consistent with our previous data (Figure 2.6). I also noticed an increase in total LRP6 levels in HEYA8 cells expressing hBetaglycan-S534A-HA/TβRIII-S534A (CS only betaglycan) compared to TβRIII-ΔGAG, with no change in phospo-LRP6 levels (Figure 3.5H). Between TβRIII-ΔGAG (no GAG chains), TβRIII-S534A (CS only chains) and TβRIII-S545A (Predominately HS chains), I find that, when HS chains are attached to betaglycan's core, they reduce both LRP6 and phospho-LRP6 levels (Figure 3.5H). Individual functions for betaglycan's HS (TβRIII-S545A) and CS chains (TβRIII-S534A)(Figure 3.5H) are consistent with our previous findings (Figure 2.6). Future studies to elucidate betaglycan's individual GAG chain roles in Wnt signaling include identifying betaglycan's GAG chain effects on β-catenin cytoplasmic accumulation/transcriptional activity and on soluble betaglycan pools, which have been shown to impact cell signaling/ligand availability (Sections 1.1iii, 2.5 and Appendix A).

3.5. Characterization of pCMV2-mSDC1-ΔCS-Flag

Using SDM, I inserted serine-to-alanine point mutations in mSDC1-FL-Flag construct at serines 207 and 217 to generate SDC1 containing CS chains exclusively (pCMV2-mSDC1-ΔCS-Flag) (Figures 3.2 and 3.6A). PCR amplification of pCMV2-

mSDC1-ΔCS-Flag produced a satisfactory amount of amplification product (Figure 3.6B) for sequence confirmation and transient expression analysis in HEK293 cells. While I observed SDC1 core protein and GAG chains for mSDC1-FL-Flag, mSDC1-ΔCS-Flag (Figure 3.6C) and mSDC1-ΔHS-His (Figure 3.6D), I could not confirm successful transfection of mSDC1-ΔGAG (data not shown), indicating further optimization of our transfection techniques is needed. Successful transient expression of all SDC1 constructs and modified SDC1 characterization, as performed for betaglycan, will lay a strong foundation for identification of new roles for DMTPs in cell biology.

3.6. Conclusions

Here I outline generation and initial characterization of three new DMTP constructs, hBetaglycan-S534A-HA, hBetaglycan-S545A-HA and mSDC1-ΔCS-Flag. In combination with full-length and GAG-less DMTP forms, the modified forms of betaglycan are used in the following chapter to shed light on individual functions for HS and CS chains in cell signaling and cancer cell biology. Experiments conducted in Chapter 4 can also be performed using the modified forms of SDC1 to identify the broad impact of HS and CS chains on proteoglycans in regulating ligand availability and cellular responses.

Table 3.1. Primer sequences for SDM of Betaglycan and SDC1

Plasmid Construct	Primers
pcDNA3.1(+)- and pHIV-dTomato-hBetaglycan-S534A-HA	F: 5'-GAAGATCTGGAG**TCA**GGTGATAATGGA-3' R*: 5'-TCCATTATCACCT**GAC**TCCAGATCTTC-3'
pcDNA3.1(+)- and pHIV-dTomato-hBetaglycan-S545A-HA	F: 5'-GAAGATCTGGAG**GCA**GGTGATAATGGA-3' R*: 5'-TCCATTATCACC**TGC**CTCCAGATCTTC-3'
pCMV2-mSDC1-ΔCS-Flag (S207A)	F: 5'-CGCAGGAGAGGGC**GCT**GGAGAACAAGAC-3' R*: 5'-GTCTTGTTCTCC**AGC**GCCCTCTCCTGCG-3'
pCMV2-mSDC1-ΔCS-Flag (S217A)	F: 5'-ACAAGACTTCACCTTTGAAACA**GCT**GGGGAGAACACAG-3' R*: 5'-CTGTGTTCTCCCC**AGC**TGTTTCAAAGGTGAAGTCTTGT-3'

5’-
MTSHYVIAIFALMSSCLATAGPYPYDVPDEPGALCELSPVSASHPVQALMESFTV
LSGCASRGTTGLPQEVHVLNLRTAGQGPGQLQREVTLHLNPISSVHIHHKSVVFL
LNSPHPVWHLKTERLATGVSRLFLVSEGSVVQFSSANFSLTAETEERNFPHGNEH
LLNWARKEYGAVTSFTELKIARNIYIKVGEDQVFPPKCNIGKNFLSLNYLAEYLQ
PKAAEGCVMSSQPQNEEVHIIELITPNSNPYSAFQVDITIDIRPSQEDLEVVKNLILI
LKCKKSVNWVIKSFDVKGSLKIIAPNSIGFGKESERSMTMTKSIRDDIPSTQGNLV
KWALDNGYSPITSYTMAPVANRFHLRLENNAEEMGDEEVHTIPPELRILLDPGAL
PALQNPPIRGGEGQNGGLPFPFPDISRRVWNEEGEDGLPRPKDPVIPSIQLFPGLRE
PEEVQGSVDIALSVKCDNEKMIVAVEKDSFQASGYSGMDVTLLDPTCKAKMNG
THFVLESPLNGCGTRPRWSALDGVVYYNSIVIQVPALGDSSGWPDGYEDLESGD
NGFPGDMDEGDASLFTRPEIVVFNCSLQQVRNPSSFQEQPHGNITFNMELYNTDL
FLVPSQGVFSVPENGHVYVEVSVTKAEQELGFAIQTCFISPYSNPDRMSHYTIIENI
CPKDESVKFYSPKRVHFPIPQADMDKKRFSFVFKPVFNTSLLFLQCELTLCTKME
KHPQKLPKCVPPDEACTSLDASIIWAMMQNKKTFTKPLAVIHHEAESKEKGPSM
KEPNPISPPIFHGLDTLTVMGIAFAAFVIGALLTGALWYIYSHTGETAGRQQVPTS
PPASENSSAAHSIGSTQSTPCSSSST-3’

Figure 3.1. Human betaglycan-FL-HA amino acid sequence with indicated serines for HS (Serine 534 in red) or CS (Serine 545 in blue) GAG chain attachment in the pcDNA3.1(+) plasmid backbone. Serine-to-alanine point mutations were made at either (pcDNA3.1(+)- and pHIV-dTomato-hBetaglycan-S534A-HA and S545A-HA) or both (pcDNA3.1(+)- and pHIV-dTomato-hBetaglycan-ΔGAG-HA) serine residues to generate all hBetaglycan constructs. Green highlighted region indicates the amino acid sequence for the HA tag inserted within hBetaglycan’s coding region as generated previously in (44).

5’-
MRRAALWLWLCALALRLQPALPQIVAVNVPPEDQDGSGDDSDNFSGSGTGALP
DTLSRQTPSTWKDVWLLTATPTAPEPTSSNTETAFTSVLPAGEKPEEGEPVLHVE
AEPGFTARDKEKEVTTRPRETVQLPITQRASTVRVTTAQAAVTSHPHGGMQPGL
HETSAPTAPGQPDHQPPRVEGGGTSVIKEVVEDGTANQLPAGEGSGEQDFTFETS
GENTAVAAVEPGLRNQPPVDEGATGASQSLLDRKEVLGGVIAGGLVGLIFAVCL
VAFMLYRMKKKDEGSYSLEEPKQANGGAYQKPTKQEEFYA-3’

Figure 3.2. Mouse SDC1 amino acid sequence with indicated serines for HS (Serines 37, 45, 47 in red) or CS (Serines 207 and 217 in blue) GAG chain attachment in the pCMV2 plasmid backbone. Serine-to-alanine point mutations were made at serine residues 207 and 217 to generate the pCMV2-mSDC1-ΔCS-Flag construct.

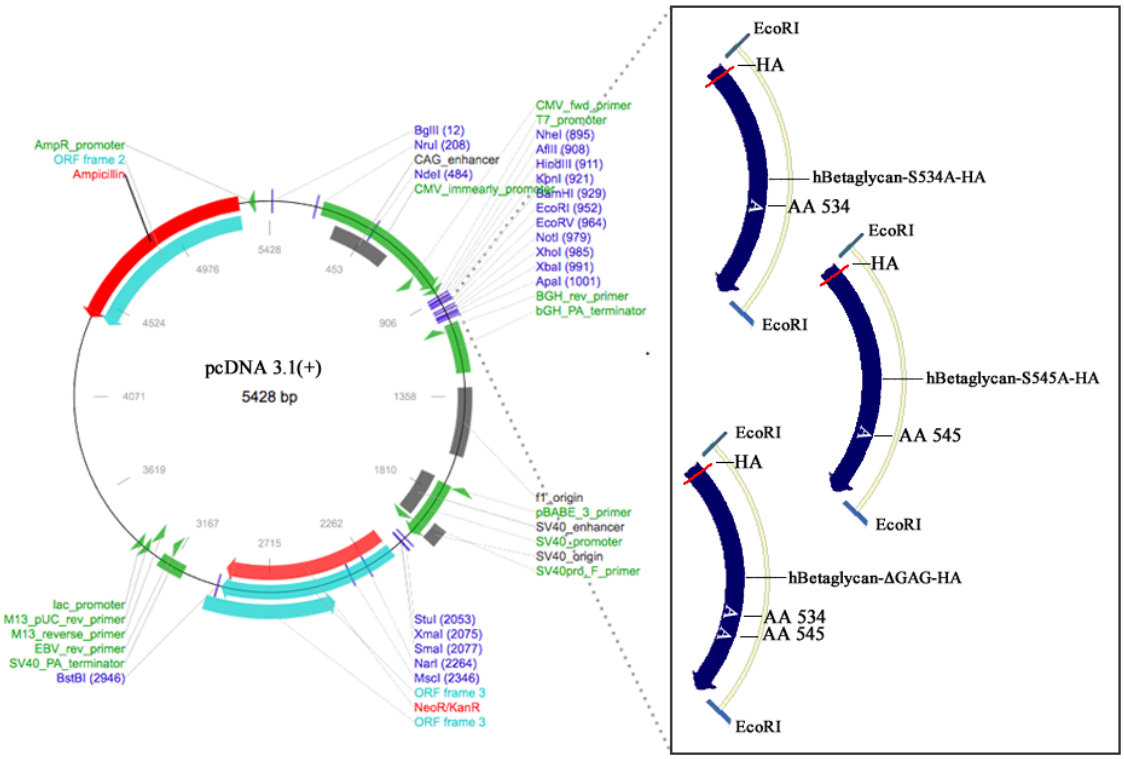

Figure 3.3. pcDNA3.1(+)-hBetaglycan-S534A-HA, -S545A-HA, ΔGAG-HA plasmid maps. Serine-to-alanine point mutations (indicated by white "A") were introduced within the hBetaglycan-FL-HA construct at the indicated amino acid. hBetaglycan-FL-HA was inserted at the EcoRI site of the pcDNA3.1(+) plasmid by (130).

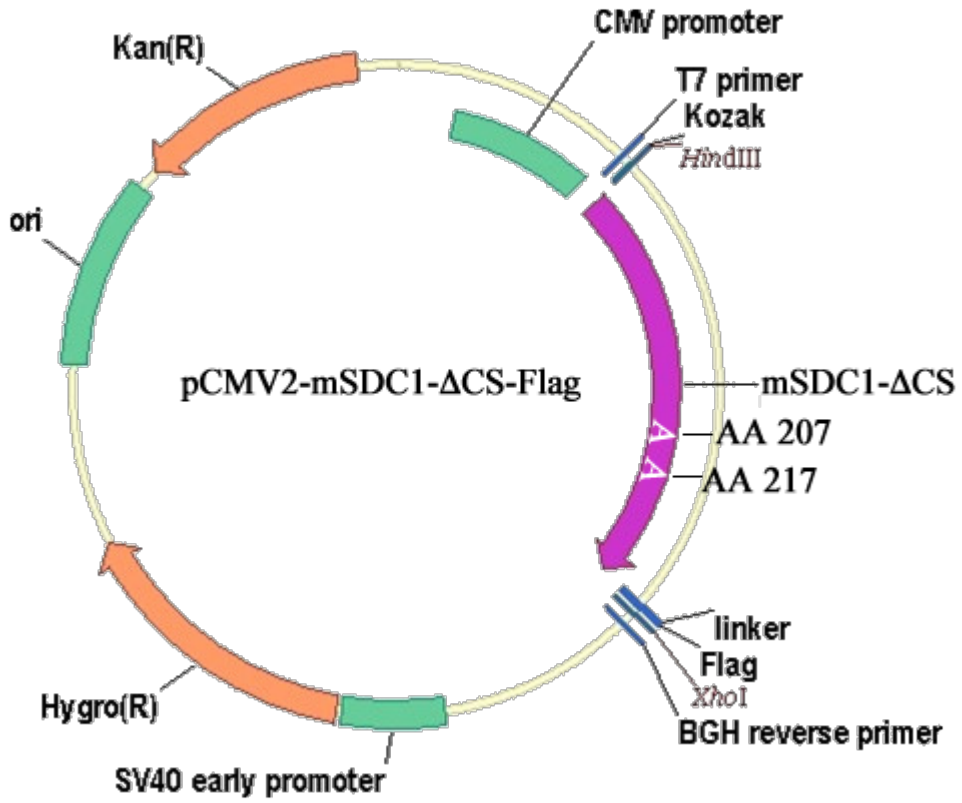

Figure 3.4. pCMV2-mSDC1-ΔCS-Flag plasmid map. To generate pCMV2-mSDC1-ΔCS-Flag, serine-to-alanine point mutations (indicated by white "A") were introduced within the pCMV2-mSDC1-FL-Flag construct at amino acids 207 (AA 207) and 217 (AA 217), the CS GAG chain attachment sites.

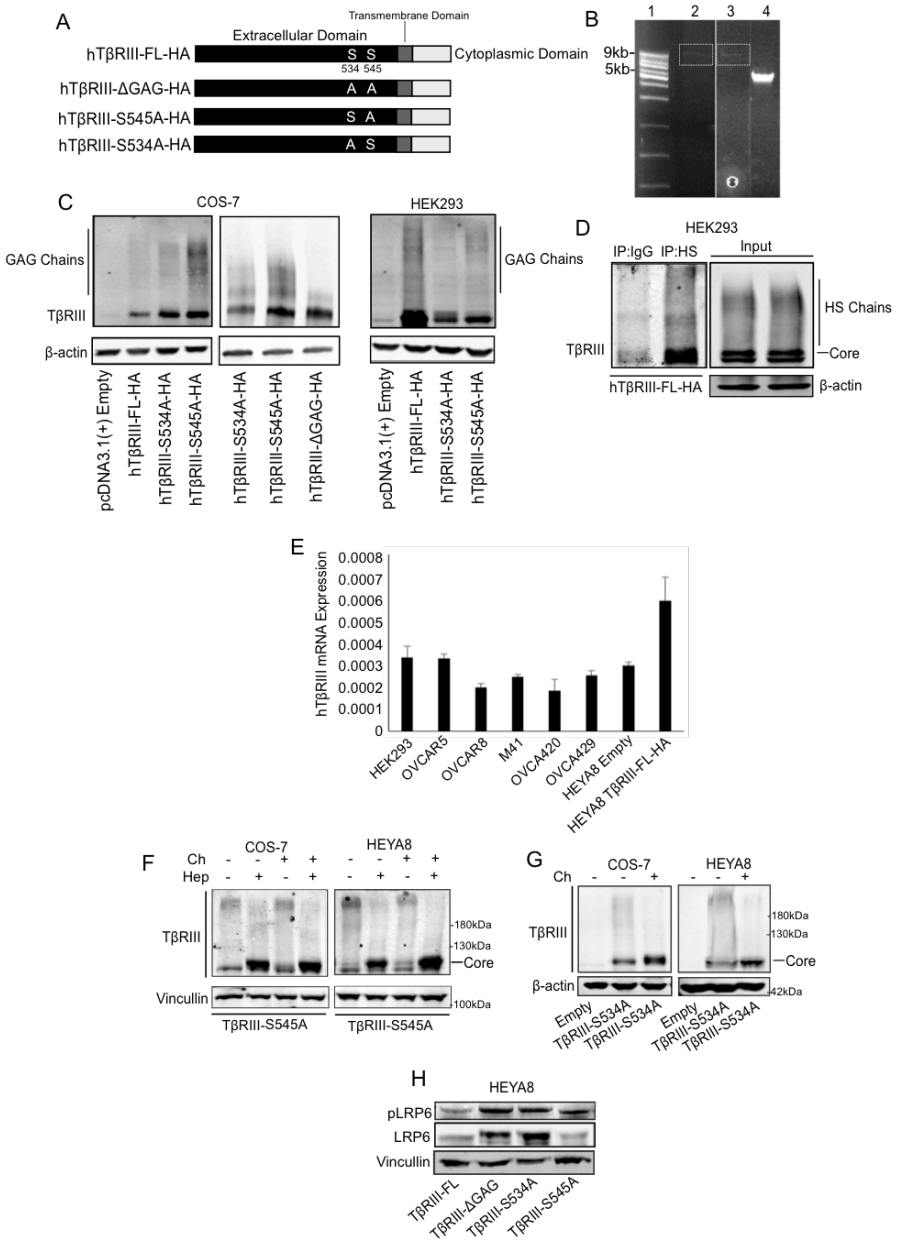

Figure 3.5. Characterization of human betaglycan-S534A-HA and -S545A-HA. (A) Serine-to-alanine point mutation at serine 534, serine 545 or both in human betaglycan for SDM of betaglycan containing either CS chains exclusively (hBetaglycan-S534A-HA), betaglycan containing predominately HS chains (hBetaglycan-S545A-HA) or betaglycan containing no GAG chains (hTβRIII-ΔGAG-HA), respectively. (B) PCR amplification of both hBetaglycan-S534A-HA (lane 2) and hBetaglycan-S545A-HA (lane 3) constructs. Lane 1 = DNA ladder, Lane 4 = QuikChange pWhitescript 4.5-kb Control. (C) Transient expression of 1 μg Empty (control) or betaglycan constructs pHIV-dTomato-hBetaglycan-FL-HA, -S534A-HA, -S545A-HA and -ΔGAG-HA in both COS-7 and HEK293 cells. Lysates were immunoblotted for TβRIII and β-actin. (D) Betaglycan

immunoprecipitation overnight with 6 μg of an anti-chicken Heparan Sulfate Proteoglycan antibody (#33 or 33-2-s, Developmental Studies Hybridoma Bank, Iowa City, IA) in betaglycan-expressing HEK293 cells. Lysates were immunoblotted for TβRIII and β-actin. (E) TβRIII mRNA expression by qRT-PCR analysis to detect endogenous TβRIII levels in seven ovarian cancer epithelial cell lines including HEYA8 cells stably expressing hBetaglycan-FL-HA (HEYA8 TβRIII-FL-HA) (positive control). Quantitations represent the average of one independent biological trial conducted in triplicate. (F-G) Enzymatic digestion of betaglycan's CS and HS chains using (F) 100 mU ml^{-1} chondroitinase ABC from *Proteus vulgaris* (#C3667-5UN, Santa Cruz) (Ch) and 50 ng ml^{-1} recombinant P. heparinus Heparinase III Protein (Hp) (R&D, #6145-GH-010) or (G) 100 mU ml^{-1} chondroitinase (Ch) alone for 2hr in both COS-7 and HEYA8 cells stably expressing the indicated hBetalgycan mutant. Lysates were immunoblotted for TβRIII and vincullin. (H) Western blot analysis of phospho-LRP6, LRP6 and Vincullin levels in HEYA8 cells lines stably expressing modified betaglycan forms.

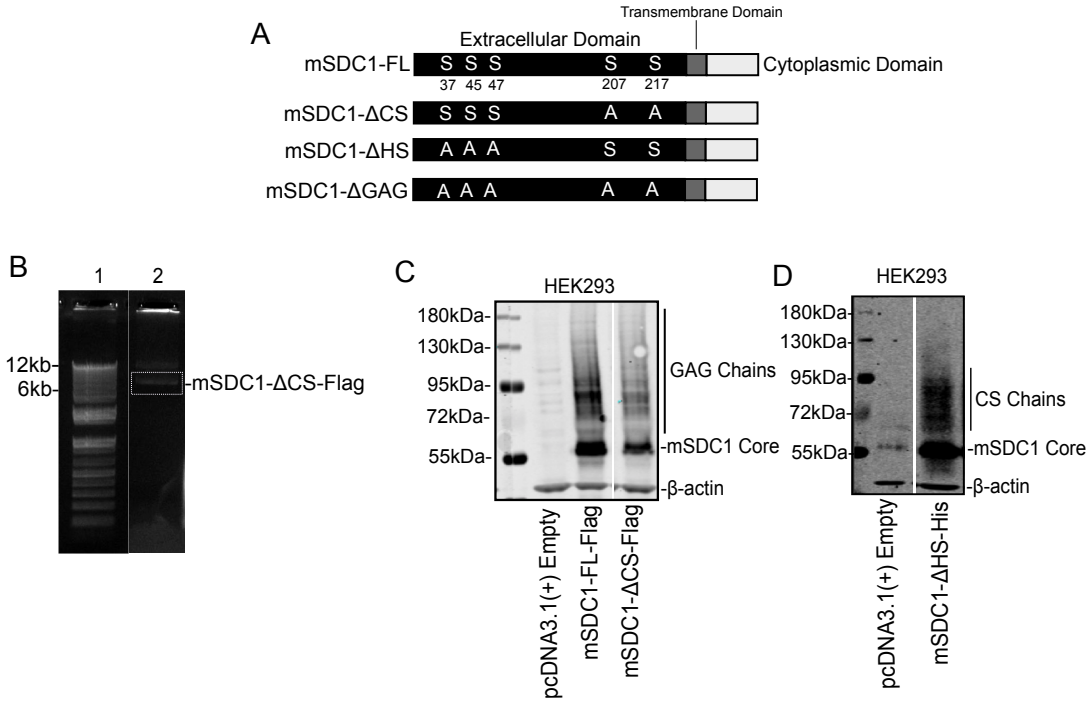

Figure 3.6. Characterization of pCMV2-mSDC1-ΔCS-Flag. (A) Serine-to-alanine point mutations at serines 37/45/47, serines 207/217 or all five serines in murine SDC1 for SDM of SDC1 containing either CS chains exclusively (mSDC1-ΔHS), SDC1 containing only HS chains (mSDC1-ΔCS) or SDC1 containing no GAG chains (mSDC1-ΔGAG), respectively. (B) PCR amplification of mSDC1-ΔCS (lane 2). Lane 1 = DNA ladder. (C-D) Transient expression of 1 μg Empty (control) and mSDC1 constructs (C) pCMV2-mSDC1-FL and -ΔCS-Flag or (D) mSDC1-ΔHS-His in HEK293 cells. Lysates were immunoblotted for SDC1 (#sc-390791, Santa Cruz) and β-actin.

CHAPTER 4

UNIQUE ROLES FOR INDIVIDUAL BETAGLYCAN GAG CHAINS IN CELL SIGNALING AND CANCER CELL BIOLOGY[3]

[3] Jenkins, L. M., Horst, B., Lancaster, C. L., and Mythreye, K. (2017). Dually modified transmembrane proteoglycans in development and disease. *Cytokine & growth factor reviews.* In press.

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4.1. Introduction

Tight control of growth factor/ligand availability at the cell surface is crucial to downstream signaling in both cancer and various other diseases/cell phenotypes(172-174). Growth factors frequently utilized by cancer cells include, but are not limited to, FGF, Wingless (Wnt), BMP/GDF, Activin and TGF-β(9,33,87,100,113,138,175-185). These growth factors can interact with DMTPs to either promote or suppress signaling. One general mechanism for increasing signal reception is by concentrating ligands near their cognate signaling receptors to activate downstream signaling. Specifically, GAG chains on DMTPs could allosterically regulate and/or oligomerize soluble ligands, serve as guides for bringing growth factors and receptors into close proximity and/or provide proper alignment for productive binding to occur(186). In contrast, sequestering ligands away from their signaling receptors and/or disrupting receptor-receptor interactions could promote DMTP-mediated cell signal suppression. DMTPs could also serve as growth factor hubs, able to release growth factors at specific times for utilization by a subset of cells in a context dependent manner.

By analyzing individual roles for HS and CS chains on DMTPs in growth factor signaling we can develop a framework to understand the biological impact DMTPs and their GAG chains have on ligand availability and signaling. Most importantly, this new knowledge could lead to the development of novel therapeutics to control cancer and other disease processes. Here, I identify new roles for betaglycan's individual GAG chains in both TGFβ superfamily and FGF signaling as well as in cancer cell behavior.

4.1.i. Betaglycan Roles in TGF-β Signaling

To initiate signaling, TGF-β members, including BMP and Activin, interact with two types of receptors (type I and II) to transduce signals via the Smad family signal transducers(33,185,187,188). Upon ligand binding and receptor activation, the receptor complex phosphorylates the C-terminus of the receptor-regulated Smad proteins (R-Smads, Smad1, 5 and 8 for BMP signaling and Smad2 and 3 for TGF-β signaling). Activated R-Smads then interact with the common partner Smad4 and accumulate in the nucleus, where the Smad complex directly binds defined elements on DNA and regulates target gene expression(33,185,187,188).

Independent of its GAG chains(127), betaglycan's ECD can bind all three TGF-β isoforms, BMP and Activin(33,189-191). Several studies suggest membrane betaglycan's primary function in TGF-β signaling is to "present" ligands, such as TGF-β2, to the Type II TGF-β receptor to activate downstream signaling(187,191-193). In fibroblasts, for example, membrane betaglycan mediates TGF-β signaling by presenting ligands and trafficking the associated TGF-β receptors(187). This coreceptor role, however, does not fully encompass betaglycan's impact on the TGF-β signaling pathway as sBetaglycan inhibits TGF-β signaling through ligand binding competition with membrane betaglycan in a variety of cell types (Figure 4.1 and (86,194,195)). Whether the impact of betaglycan in cancer is via direct TGF-β-mediated mechanisms has been poorly demonstrated/tested, as domain specific functions of betaglycan's ECD *in vivo* have not been investigated in-depth. However, upon loss of betaglycan, cancer cells do become less sensitive to TGF-β-mediated cancer growth and migration, indicating that betaglycan can impact TGFβ-dependent regulation of cancer cell behavior (Figure 4.1 and (193)). More recently,

betaglycan has been shown to tether TGF-β1 to the surface of cancer cell exosomes, which help cancer cells grow and spread as they drive TGF-β-induced differentiation of cancer-associated fibroblasts into myofibroblasts(196).

To regulate BMP signaling, studies have shown that betaglycan's core domain directly binds BMP2, although with lower affinity than TGF-β(33), and BMPs' cognate receptors ALK3 and ALK6 (Figure 4.1 and (33)). Not only does betaglycan interact with BMP2, independent of its GAG chains(33), betaglycan's core domain also directly binds BMP4, BMP7 and GDF-5 to enhance ligand binding to ALK3 and ALK6(33). This binding, in turn, promotes EMT of AV endothelial cells during development. In prostate cancer, betaglycan knockdown correlated with a significant decrease in BMP2 mediated Smad1 phosphorylation, indicating critical roles for betaglycan in facilitating BMP functions in both cancerous and non-cancerous cells(33).

In addition to betaglycan's ability to affect BMP induced cell biology through direct BMP binding, betaglycan's core domain also impinges on BMP and TGF-β/Activin signaling through high affinity core domain interactions with another TGF-β family member: Inhibin (Figure 4.1 and (189,197)). Upon binding Inhibin, betaglycan promotes Inhibin interactions with ActRII, ActRIIB and/or BMPRII type II receptors(189,198). This Inhibin-betaglycan-type II receptor complex leads to prevention of Activin and BMP induced type I-type II receptor interactions for signaling(189,198,199). Since Inhibin binds with low affinity to type II receptors in the absence of betaglycan, the core domain of betaglycan plays a central role in potentiating Inhibin antagonism of Activin and BMP signaling (198,199).

4.1.ii. FGF Signaling and Betaglycan

FGF glycoproteins, via their FGF receptors, regulate cancer cell proliferation, apoptosis, angiogenesis, EMT, motility and invasion and can also contribute to chemoresistance(174,200). The 18 members of the FGF family initiate their signaling cascade through four highly conserved transmembrane tyrosine kinase receptors (FGFR1-4). To access these receptors, secreted FGF ligands are released from ECM HSPGs, via heparinase and protease digestions(201,202), so that they can bind to FGFRs and cell surface HSPGs which aid in FGF receptor stabilization and downstream activation of multiple signaling pathways(201,203). In both neuroblastoma and osteoblasts, betaglycan's HS chains are required for FGF activity (9,131). In neuroblastoma, betaglycan's GAG chains bind FGF2 and FGFR1 to activate MAPK/ERK pathways and promote neuronal differentiation. This signal induction causes a significant decrease in neuroblastoma cell proliferation *in vitro* and tumor growth and metastasis *in vivo*, supporting betaglycan's tumor suppressive role in cancer(9).

4.2. Experimental Procedures

Western Blotting–Western blotting was performed using standard techniques as described previously (90,93,135). All antibodies were obtained from Cell Signaling and include anti-rabbit phospho-p44/42/MAPK/Erk1/2 (#3179), anti-rabbit phospho-SMAD1/5 (#9516), anti-rabbit SMAD1 (#6944) and anti-rabbit p44/42/MAPK/Erk1/2 (#4695).

Cell Viability Assay–Following stable expression of modified betaglycan in HEYA8 cells, the viability of HEYA8 cells was determined using MTT/Thiazoyl Blue Tetrazolium Bromide (#298-93-1, VWR, Radnor, PA), Briefly, 4K HEYA8 cells were

plated in a 96-well dish and incubated for 48 hr. Media was then removed and 10 μl of 5 mg/ml MTT added to each well. Cells were incubated for 4 hr, MTT was removed and cells were resuspended in DMSO. Cell viability was measured at 570 nm with a microplate reader (Synergy H1 Hybrid Multi-Mode Reader, BioTek Instruments, Inc., Winooski, VT).

Cell Growth Curves–HEYA8 cells (20K) were seeded into 12-well dishes. On the indicated day, cells were trypsinized and counted. Twelve measurements between two biologically independent wells/condition were counted averaged +/- SEM.

Matrigel Invasion Analysis–Invasion assays were performed using 24-well transwells (Greiner Bio-One, Kremsmünster, Austria; ThinCerts^{TM}, 24 well 8.0 μm) coated with 400 μg/ml MatriGel (BD Biosciences #3248404). Cells (20K) in serum free media were seeded in the upper chamber and allowed to invade for 24 hr toward serum media in the lower chamber. Filters were stained with Three Step Stain (Richard-Allan Scientific, San Diego, CA, USA). Filters were removed and mounted onto glass slides. Cells on the filter were counted using an Olympus DP21 microscope at x10 magnification.

4.3. Effect of betaglycan's individual GAG chains on TGFβ superfamily and FGF signaling

To determine roles for betaglycan's individual GAG chains on TGFβ superfamily signaling, I have analyzed phosphorylated SMAD1/5 and total SMAD1 basal levels in our HEYA8 cells stably expressing our modified betaglycan forms. Compared to full-length betaglycan, betaglycan-ΔGAG (TβRIII-ΔGAG) increases both phosphorylated and total SMAD1 levels (Figure 4.2A). When CS chains are added to betaglycan (TβRIII-

ΔGAG versus TβRIII-S534A), phosphorylated SMAD1/5 levels increase while HS chains on betaglycan (TβRIII-S545A versus TβRIII-ΔGAG or TβRIII-S534A) suppress SMAD1/5 phosphorylation and reduce total SMAD1 levels (Figure 4.2A). These data indicate potentially opposing roles for betaglycan's GAG chains in BMP signaling and lay the foundation for an in-depth analysis of betaglycan's individual GAG chain effects on both BMP signaling (SMAD1/5) and TGFβ signaling (SMAD2/3) in the future.

I have also initiated studies to determine the impact of betaglycan's individual chains on basal FGF/ERK signaling in HEYA8 ovarian cancer cells. My preliminary findings indicate that hBetaglycan-S534A-HA (TβRIII-S534A) may suppress ERK phosphorylation (TβRIII-S534A versus TβRIII-ΔGAG) while the HS chains can enhance ERK phosphorylation (TβRIII-S545A versus TβRIII-S534A and in (9,100)) in our HEYA8 cells expressing modified betaglycan (Figure 4.2B).

An in-depth study on individual functions for GAG chains on DMTPs, as they relate to FGF signaling and FGF-induced cancer cell biology in different cancer types, is warranted as it could reveal why DMTPs and sDMTPs exhibit both tumor-promoting and tumor-suppressive roles in FGF-mediated cancer progression.

4.4. Impact of betaglycan's individual GAG chains in regulating cancer cell behavior

As outlined in Section 1.3, betaglycan is a crucial regulator of cancer progression with defined roles in apoptosis, adhesion, angiogenesis, proliferation, migration, invasion and metastasis *in vitro* and *in vivo*(2,9,39,52,86-96). As a strong predictor of overall and recurrence-free patient-survival in multiple cancers (86,90,100), it is crucial that we

elucidate functions for betaglycan and its individual GAG chains in regulating key cancer phenotypes involved in averting current therapeutic strategies.

To initiate these studies, we first analyzed modified betaglycan's effects on HEYA8 ovarian cancer cell viability via a MTT assay. We find that betaglycan's CS chains have no significant impact on HEYA8 cell viability (TβRIII-ΔGAG versus TβRIII-S534A). Betaglycan's HS chains, however, reduce cell viability (TβRIII-S534A versus TβRIII-S545A), indicating a potential role for betaglycan's HS chain in reducing ovarian cancer cell viability (Figure 4.2C). In line with this data, in both HEK293 and HEYA8 cells stably expressing modified forms of betaglycan, we find that betaglycan's HS chains suppress cell proliferation (TβRIII-ΔGAG versus TβRIII-S534A) while the CS chains do not (TβRIII-ΔGAG versus TβRIII-S534A) (Figure 4.2D). To determine whether betaglycan's individual chains alter ovarian cancer cell invasion, we performed a MatriGel invasion assay using betaglycan-expressing HEYA8 cells and find that attachment of CS chains to betaglycan's core suppresses cancer cell invasion (TβRIII-ΔGAG versus TβRIII-S534A) (Figure 4.2E) with no additional impact on invasion from betaglycan's HS chains (Figure 4.2E).

4.5. Conclusions

Taken together, these data reveal unique, and at times opposing, roles for betaglycan's HS and CS chains in cell signaling and biology. In BMP signaling, for example, HS-betaglycan (TβRIII-S545A) reduces SMAD1/5 phosphorylation while CS-betaglycan (TβRIII-S534A) enhances SMAD1/5 phosphorylation. During FGF signaling, contrarily, TβRIII-S534A suppresses ERK phosphorylation while TβRIII-S545A enhances phosphorylation of ERK. Moreover, these opposing effects on signal transduction may contribute to the selectively of CS-betaglycan and HS-betaglycan in

controlling different cancer cell phenotypes. My bioassays revealed, for instance, that betaglycan's HS chains reduced both ovarian cancer cell viability and proliferation with little to no contribution from betaglycan's CS chains. However, CS-betaglycan, independent of betaglycan's HS chains, were able to suppress cancer cell invasion, highlighting the importance of different chain types, on a single proteoglycan, in the regulation of aberrant cell biology.

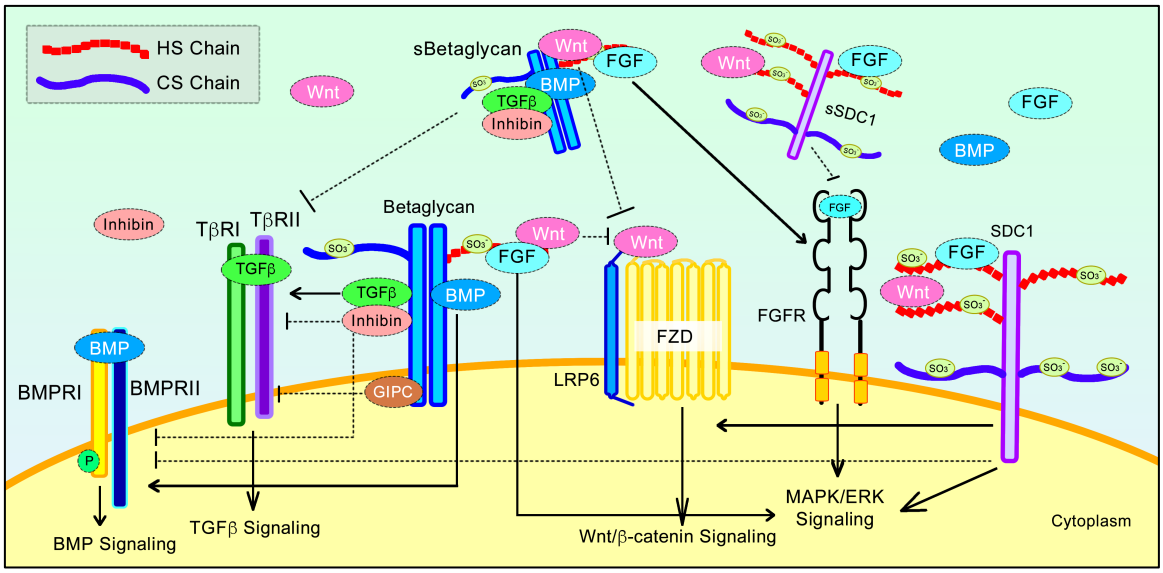

Figure 4.1. DMTPs in growth factor signaling pathways. DMTPs betaglycan and SDC1 bind growth factors either through their GAG chains or core protein and then regulate ligand function through distinct mechanisms. Growth factors bound to membrane DMTPs are localized to the cell surface and are either presented to their associated signaling receptors (→) or sequestered away to prevent signal transduction (---|). Additionally, soluble DMTP forms, generated by ectodomain shedding, bind ligands and either enhance or prevent signal complex formation. Abbreviations: *BMP*: Bone Morphogenetic Protein, *BMPR:* Bone Morphogenetic Protein Receptor, *CS*: Chondroitin Sulfate, *ERK:* Extracellular Signal-regulated Kinase, *FGF*: Fibroblast Growth Factor, *FGFR:* Fibroblast Growth Factor Receptor, *FZD*: Frizzled, *GIPC*: GAIP-interacting protein C terminus, *HS*: Heparan Sulfate, *LRP6:* LDL Receptor Related Protein 6, *MAPK*: Mitogen-activated protein kinase, sBetaglycan*:* Soluble/shed betaglycan, *sSDC1:* Soluble/shed SDC1, *TGFβ:* Transforming Growth Factor β, *TβRI, RII*: Transforming Growth Factor β Receptors I and II, *Wnt*: Wingless.

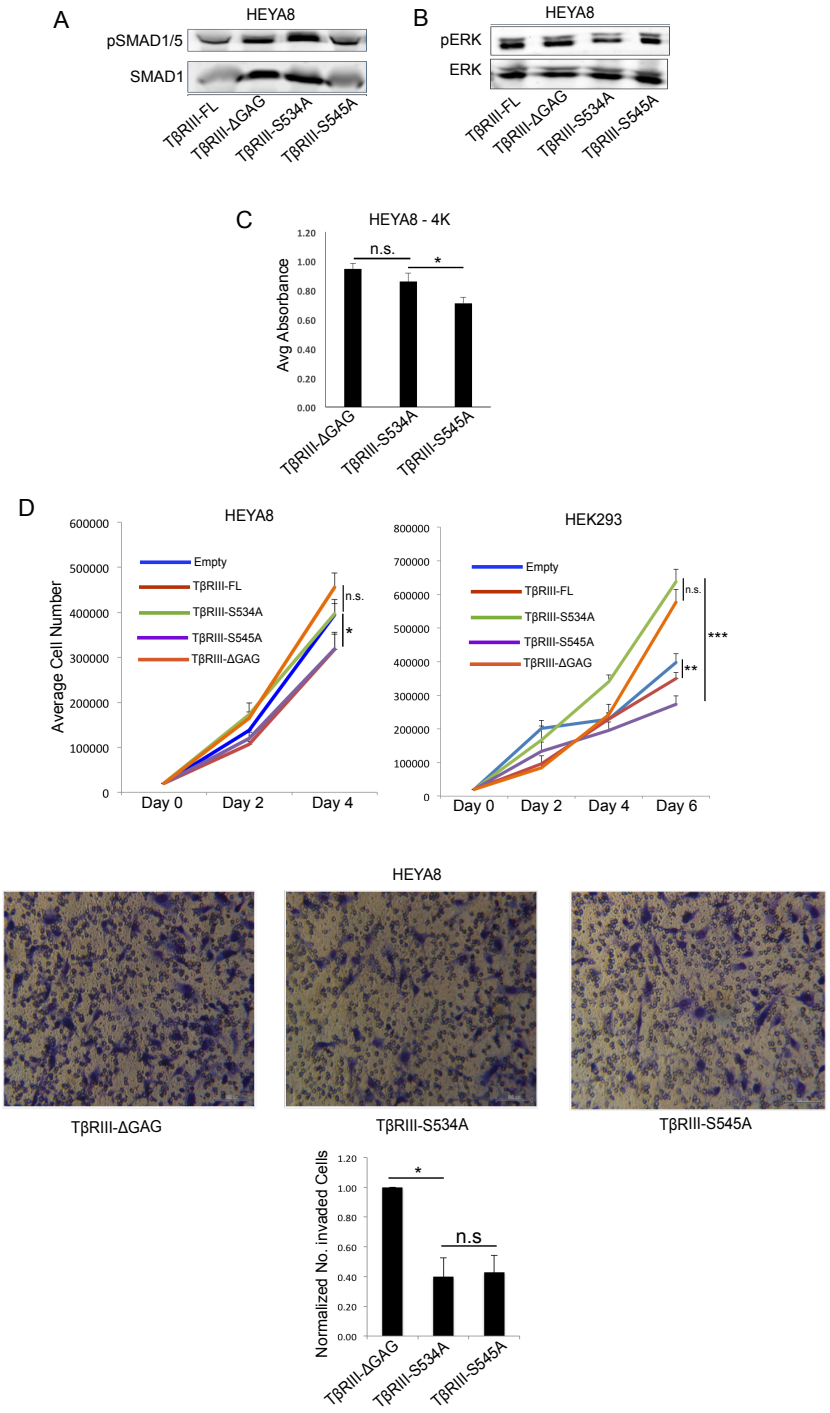

Figure 4.2. Roles for betaglycan's individual GAG chains in cell signaling and cancer cell behavior. HEK293 or HEYA8 (ovarian cancer) cells expressing the indicated TβRIII/Betaglycan mutant or Empty (control) were analyzed as follows: (A) Western blot analysis of phospho-SMAD1/5, total SMAD1/5, phospho-ERK1/2 and total ERK1/2 levels. (B) Cancer cell viability/MTT/Thiazoyl Blue Tetrazolium Bromide assay of 4 x

10^3 cells plated in a 96-well dish and incubated for 48 hr. Cell viability was measured at 570 nm with a microplate reader (Experimental Procedures). (C) Cell growth curves from 2 x 10^4 cells for indicated number of days. Quantitations represent the average of two independent biological trials each conducted in sextuplet. (D) Transwell invasion through Matrigel measured after 24hr. All quantitative data analyzed using two-tailed Student's t-test and represent the mean±s.e.m.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

Much is known about the functions of SDC1 and betaglycan since their discoveries in 1989(34) and 1985(204) respectively, yet better definitions of the disease contexts in which the GAG chains on DMTPs are most relevant is required to improve our ability to use these DMTPs as diagnostic and prognostic markers and as biomarkers for treatment response. At the cell–ECM interface, betaglycan can control tumorigenic processes such as growth, invasion and metastasis by regulating growth factor availability through their GAG chain attachments. This dissertation work provides, for the first time, evidence of Wnt signaling regulation by TβRIII/Betaglycan through distinct functions of its HS and CS GAG chains. My biochemical studies demonstrate that HS-betaglycan (TβRIII-S545A) suppresses Wnt3a signaling, most likely via sequestering Wnt, while CS-betaglycan (TβRIII-S534A) promotes Wnt signaling in ovarian cancer cell lines. Mechanistically, pull-down assays in betaglycan-expressing cells (Figure 2.5) indicate interaction between betaglycan's GAG chains and Wnt glycoproteins that likely lead to sequestration of Wnts away from their cognate receptors and subsequent Wnt signal suppression. To further characterize individual roles for betaglycan's HS and CS chains in cancer biology, I employed an SDM protocol to generate human betaglycan mutants expressing either CS exclusively (hBetaglycan-S534A-HA) or predominately HS chains (hBetaglycan-S545A-HA) and confirmed GAG chain attachment via heparitinase and chondroitinase enzymatic digestions (Chapter 3). Using these betaglycan mutants, I have identified distinct roles for betaglycan's HS and CS chains in both BMP and FGF signaling (Chapter 4) as well as in cancer cell biology *in vitro*, with HS-betaglycan capable of suppressing epithelial cell growth/viability and CS-betaglycan suppressing cancer cell invasion *in vitro* (Chapter 4).

In addition to generating betaglycan mutants, I also generated a murine SDC1 mutant containing only HS chains (mSDC1-ΔCS-Flag). In combination with full-length, CS only and GAG-less SDC1 forms, this SDC1-ΔCS-Flag construct can be used to determine whether betaglycan's GAG chain effects on cell signaling and cancer biology are specific to betaglycan or signify broad roles for HS and CS chains on proteoglycans in regulating ligand availability and cellular responses.

To further characterize individual, context-dependent roles for GAG chains on DMTPs in cell signaling and cancer biology, several biochemical assays can be employed. First, using radiolabeled growth factors (TGFβ, Inhibin, Wnt, BMP, FGF) and cross-linking and binding studies, roles for DMTP GAG chains in proteoglycan-ligand interactions could be determined. To evaluate the effects of DMTP GAG chains on DMTP shedding, which has been shown to impact cell signal transduction (Section 1.1.ii, 2.5 and Figure A.1B), an ELISA could be performed with conditioned media (which contains soluble DMTPs) from cells expressing different DMTP forms (HS only, CS only or GAG-less). Additionally, to determine whether modifying GAG chains on betaglycan alters cell surface expression and intracellular localization, which can also impact cell signaling responses, subcellular fractionation or immunofluorescent analysis could be performed. These localization studies would also aid in delineating receptor/ligand trafficking/internalization mechanisms related to changes in DMTP GAG chain expression. Taken together, these studies would add to current knowledge on individual roles for GAG chains on a single core protein in regulating key biological phenotypes and cell signaling pathways responsible for promoting aberrant cell behavior and disease

progression. This increase in knowledge, in turn, will lead to the development of precise, context-dependent therapeutic strategies.

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APPENDIX A

BETAGLYCAN IN GROWTH FACTOR SIGNALING

Appendix Results and Discussion

This appendix includes supportive data for betaglycan roles in epithelial cell signaling and biology.

Figure A.1A. Betaglycan reduces TCF/LEF activity in IGROV ovarian cancer cells. Both SKOV3 (Figure 2.1A, left graph) and IGROV ovarian carcinoma epithelial cells express higher endogenous betaglycan levels, compared to other ovarian cancer cell lines (i.e. OVCA429). As such, to determine whether higher betaglycan levels in IGROV cells contribute to reduced Wnt signaling, as observed in SKOV3 cells (Figure 2.2C), I used shRNA to betaglycan and measured TCF/LEF activity in these cells. I find enhanced basal and Wnt induced TCF/LEF-reporter activity in shBetaglycan IGROV cells, compared to control cells (Figure A.1A), supporting observations that betaglycan is, in part, responsible for the reduced TCF/LEF activity in cells with higher endogenous betaglycan (Figure 2.2C).

Figure A.1B. Soluble CS-betaglycan enhances LRP6 phosphorylation. To support CS-betaglycan's role in enhancing Wnt-induced TCF/LEF activity in OVCA429 cells (Figure 2.6 and 3.5H), I analyzed LRP6 phosphorylation in both HEK293 and OVCA429 cells after stimulation with Wnt3a and in the presence of conditioned media (sGFP/sTβRIII-FL/sTβRIII-ΔGAG) from CHO pgsD-677 cells (express CS GAG chains only). I observed an increase in LRP6 phosphorylation in the presence of soluble CS-betaglycan compared to control cells or cells expressing GAG-less betaglycan (Figure A.1B), supporting the previously established Wnt signaling promoter role for CS-betaglycan in Wnt signaling (Figures 2.6D-E and 3.5H).

Figure A.1C. Betaglycan's GAG chains differentially affect Wnt target genes. In HEYA8 ovarian cancer cells, modified forms of betaglycan were stably expressed (Chapter 3) to analyze the individual roles betaglycan's HS and CS chains play in regulating cellular signaling events. To determine whether betaglycan's individual GAG chains alter Wnt target gene mRNA expression, qRT-PCR analysis of common Wnt target genes *axin2*, *c-myc* and *ccnd1* was performed in both HEK293 cells (confirmation of transient betaglycan GAG chain expression awaiting enzymatic digestion analysis), and HEYA8 ovarian cancer cells. I observed no significant change in basal Wnt target gene mRNA expression upon addition of CS chains to betaglycan's core domain (TβRIII-ΔGAG vs. TβRIII-S534A) with the exception of a slight increase in *axin2* mRNA expression in HEK293 cells (Figure A.1C). Interestingly, I observed an increase in basal mRNA expression levels for the majority of Wnt target genes upon addition of HS chains (TβRIII-S534A vs. TβRIII-S545A) to betaglycan's core (Figure A.1C), indicating that betaglycan's HS chains may promote transcription of certain Wnt target genes in certain cellular contexts. Further analysis of Wnt target gene activation by betaglycan's posttranslational modifications is warranted to fully elicit the impact of DMTPs on Wnt target gene activity.

Figure A.1D. GAG chains on betaglycan alter Frizzled1-betaglycan interactions. To identify a potential mechanism for betaglycan's suppressive effects on Wnt signaling activity, I performed a betaglycan-Frizzled (Fzd) proximity ligation assay in OVCA429 cells expressing either full-length betaglycan (TβRIII-FL) or GAG-less betaglycan (TβRIII-ΔGAG) (Figure A.1D). Compared to control cells, I observed a decrease in Fzd-betaglycan proximity upon Wnt3a treatment (Figure A.1D), supporting a

mechanism that allows betaglycan to sequester Wnt3a away from Fzd in order to reduce signal activation. Moreover, upon removal of betaglycan's GAG chains, I observed no decrease in betaglycan-Fzd proximity after Wnt3a stimulation, indicating betaglycan's GAG chains play a direct role in regulating Fzd-betaglycan proximity.

Figure A.1E. Betaglycan suppresses Wnt5a signaling in bladder cancer. In addition to betaglycan's roles in suppressing Wnt3a signaling, betaglycan is also able to suppress Wnt5a signaling. J82 bladder cancer cells expressing full-length betaglycan were unable to induce JNK phosphorylation to the same extent as control cells treated with Wnt5a in a time-dependent manner (Figure A.1E). These data (Figure A.1E and Chapter 2) indicate that betaglycan is capable of suppressing multiple hyperactive Wnt signaling cascades in epithelial cells.

Figure A.1F. Betaglycan interacts with Wnt5a. To determine a potential mechanism for betaglycan's suppressive effects on Wnt5a signaling, I performed co-immunoprecipitation analysis (see Chapter 2 Methods) in COS-7 cells transiently expressing full-length betaglycan and murine Wnt5a (MG50154-NF, Sino Biologicals). I observed betaglycan-Wnt5a interactions (Figure A.1F), which could potentially reduce Wnt5a signaling (as observed in Figure A.1E) if betaglycan sequesters Wnt5a from its receptors.

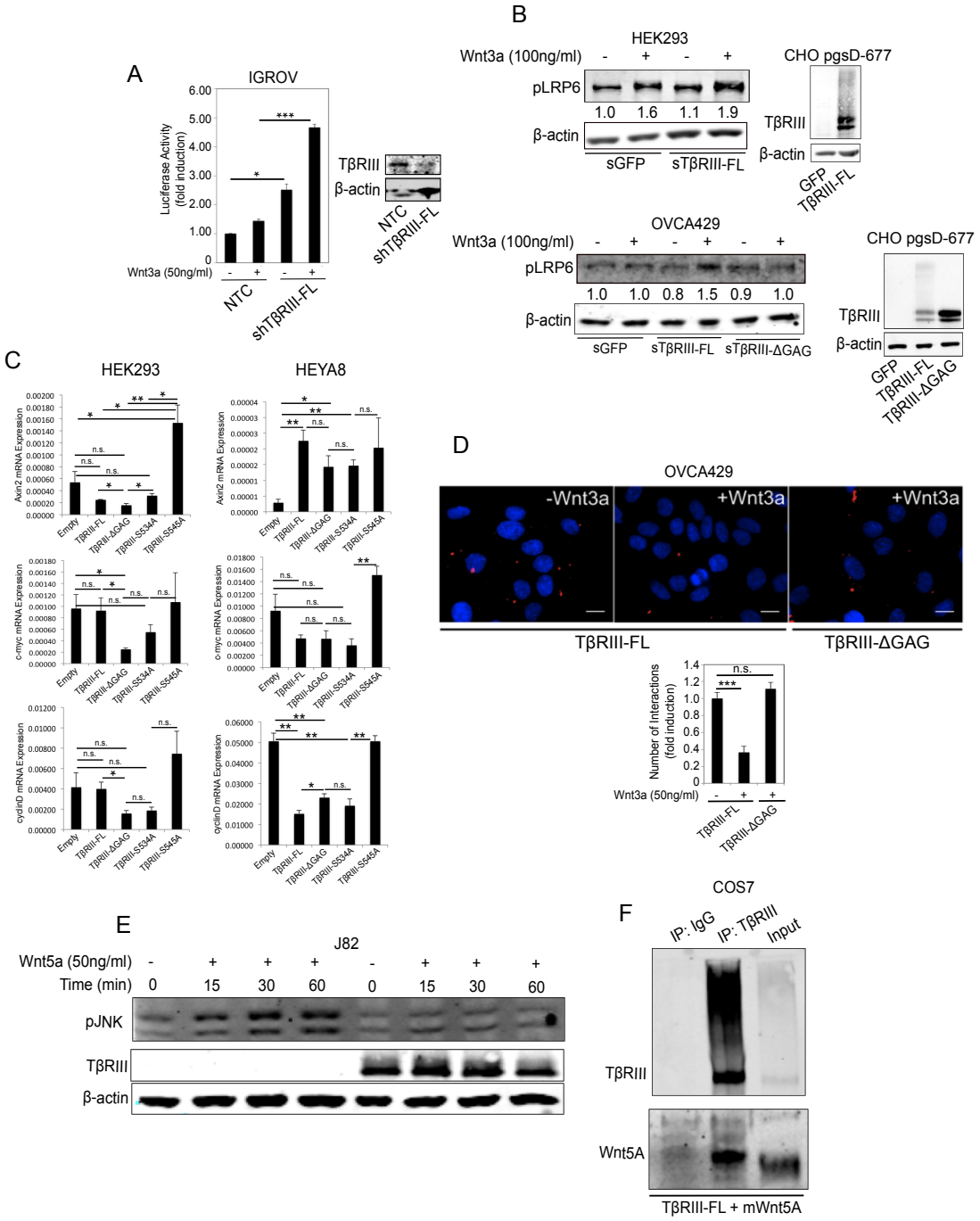

Figure A.1. Additional roles for betaglycan in Wnt signaling. (A) Indicated cells expressing either NTC (control) or shTβRIII were transfected with a Wnt-responsive luciferase reporter and SV40 control vector and left untreated or treated with 50 ng ml^{-1} Wnt3a for 24 h. Luciferase activity was then measured as described in Experimental Procedures. All values normalized to the untreated sample and represent the average of two independent biological trials each conducted in duplicate. Data analyzed using two-tailed Student's t-test and represent the mean±s.e.m. (B) Indicated cells were stimulated with 100 ng ml^{-1} Wnt3a in the presence of conditioned media (sGFP/sT β RIII-FL) from

CHO pgsD-677 cells expressing either TβRIII with CS chains exclusively (sT β RIII-FL), GAG-less T β RIII or GFP for 24 h (sGFP). Right CHO pgsD-677 graphs indicate T β RIII expression levels in these cells. Lysates were immunoblotted for phospho-LRP6, TβRIII and β-actin. (C) qRT-PCR analysis was performed in HEK293 and HEYA8 (ovarian cancer) cells expressing the indicated TβRIII/Betaglycan mutant or Empty (control) to detect mRNA expression of Wnt target genes *axin2, c-myc* and *cyclin-D.* Quantitations represent the average of one independent biological trial conducted in triplicate. Data analyzed using two-tailed Student's t-test and represent the mean±s.e.m. (D) Fluorescent images of proximity ligation in OVCA429 ovarian cancer cells between full length TβRIII or TβRIII-ΔGAG with endogenous Frizzled1 (Fzd1), with or without 1 h 50 ng ml^{-1} Wnt3a treatment. Quantification of the interaction compared with control cells is presented. N ≥ 50 cells/condition. (E) Western analysis of 50 ng ml^{-1} Wnt5a treatment at indicated times in J82 bladder cancer cells. Lysates were immunoblotted for phospho-JNK (#9255, Cell Signal), TβRIII and β-actin. (F) COS-7 cells transiently expressing full length TβRIII and murine Wnt5a-HA (mWnt5a-HA) were immunoprecipitated using anti-TβRIII and immunoblotted using anti-Wnt5a (#2530, Cell Signal) and anti-TβRIII to analyze Wnt5a-TβRIII interactions as described for Wnt3a in Chapter 2 Experimental Procedures and previously in (90,93,135).

Figure A.2. Betaglycan's GAG chains differentially affect TGFβ superfamily target genes and MMP mRNA expression. To determine roles for betaglycan's GAG chains on growth factor target genes, I performed qRT-PCR in HEYA8 and HEK293 cells expressing modified forms of betaglycan and analyzed mRNA expression for TGFβ (*ID1*) and BMP (*PAI1*) target genes (Figure A.2A-B). Additionally, I analyzed mRNA levels for MMPs, which cleave betaglycan and release sBetaglycan into the extracellular environment (See Section 1.1.iii). In normal HEK293 epithelial cells, betaglycan's CS chains enhanced both *ID1* and *PAI1* mRNA expression (Figure A.2A-B, TβRIII-ΔGAG vs. TβRIII-S534A). In HEYA8 ovarian cancer epithelial cells, however, CS-betaglycan suppressed *ID1* and *PAI1* mRNA expression (Figure A.2A-B, TβRIII-ΔGAG vs. TβRIII-S534A), indicating opposing roles for CS-betaglycan in normal versus cancerous epithelial cells. Contrasting functions for HS-betaglycan were also observed in normal HEK293 cells versus cancerous HEYA8 cells. Compared to TβRIII-S534A (CS-betaglycan), betaglycan's HS chains decreased *ID1* expression in HEK293 cells but enhanced *ID1* mRNA expression in HEYA8 cells (Figure A.2A, TβRIII-S534A vs. TβRIII-S545A). A slight decrease in *PAI1* mRNA expression was observed upon addition of HS chains to betaglycan's core domain in HEK293 cells (Figure A.2B, HEK293 TβRIII-S534A vs. TβRIII-S545A) while in HEYA8 ovarian cancer cells, adding HS chains to betaglycan significantly enhanced *PAI1* mRNA expression (Figure A.2B, HEYA8 TβRIII-S534A vs. TβRIII-S545A). These data support unique and opposite roles for betaglycan's individual GAG chains in normal versus cancerous epithelial cells, as it relates to growth factor target gene activity, and warrant further exploration.

Analysis of MMP expression in HEK293 cells, upon addition of CS chains to betaglycan, revealed a significant increase in MMP10 mRNA levels but a decrease in MMP3 mRNA expression (Figure A.2C, TβRIII-ΔGAG vs. TβRIII-S534A), indicating differences in MMP target gene regulation by CS-betaglycan. I observed no significant effect on MMP mRNA expression upon attachment of HS chains to betaglycan (Figure A.2C, TβRIII-S534A vs. TβRIII-S545A), indicating HS chains on betaglycan do not affect MMP mRNA expression in normal epithelial cells. In HEYA8 cells, CS-betaglycan did not alter MMP3 or MMP10 mRNA expression while HS-betaglycan only increased MMP10 mRNA levels (Figure A.2C, TβRIII-S534A vs. TβRIII-S545A), indicating minor contributions from betaglycan's GAG chains on MMP3 and MMP10 expression/activity. Analysis of other MMPs may reveal new functions for DMTP GAG chains in regulating MMP mRNA and protein levels in cancer.

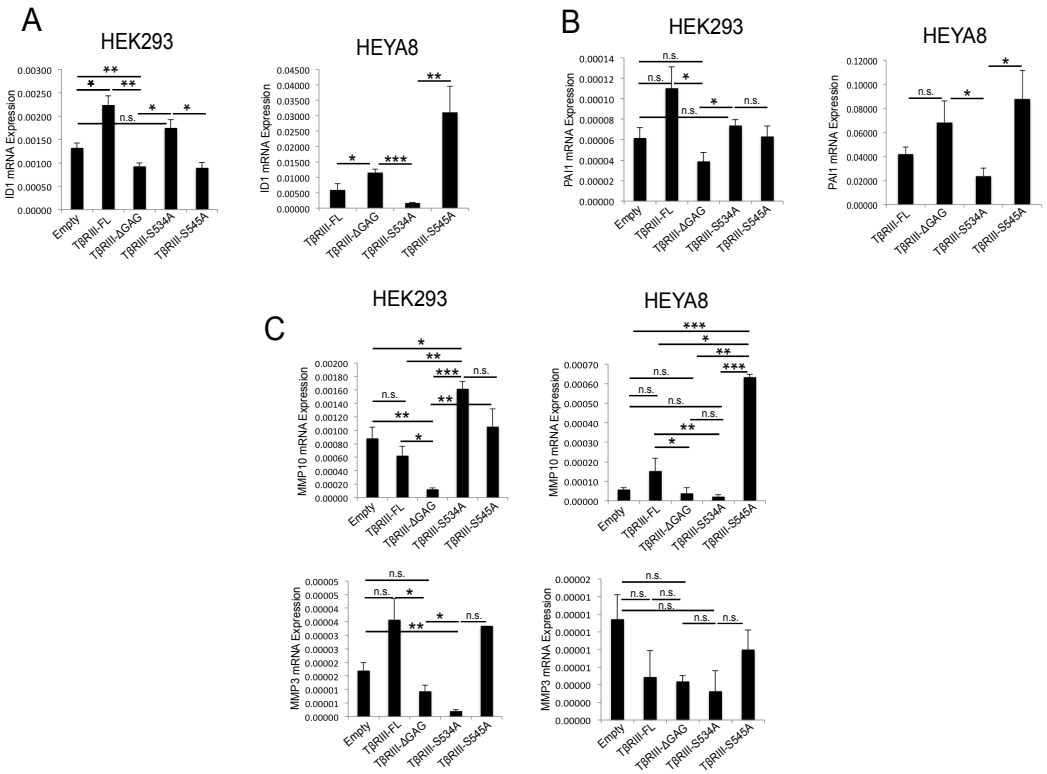

Figure A.2. Betaglycan alters matrix metalloproteinases (MMPs) and TGFβ superfamily target gene mRNA. qRT-PCR analysis was performed in HEK293 and HEYA8 (ovarian cancer) cells expressing the indicated TβRIII/Betaglycan mutant or Empty (control) to detect: (A) *ID1* (BMP target gene) mRNA expression, (B) *PAI1* (TGFβ target gene) mRNA expression or (C) *MMP3* and *MMP10* mRNA expression. Quantitations represent the average of one independent biological trial conducted in triplicate. Data analyzed using two-tailed Student's t-test and represent the mean±s.e.m.

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